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USE OF SHORT-TERM BIOASSAYS TO EVALUATE ENVIRONMENTAL IMPACT OF LAND TREATMENT OF HAZARDOUS INDUSTRIAL WASTE

by

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16. ABSTRACT

A four phase study was conducted to evaluate utility of short-term bioassays in monitoring environmental impact of land treatment of hazardous waste. During phase one, three microbial bioassays were conducted to define chronic toxic potential of each waste selected for study. Acid, base, and neutral fractions of each of three wastes studied induced genetic damage in at least two of the three bioassays.

Phase two was conducted to evaluate efficiencies of blender and soxhlet extraction procedures, as well as potential interactions between known mutagens and soil components. Results indicate that there was no appreciable difference in mutagenicity of the extract using either procedure. Using the blender procedure extraction efficiency for pure compounds added to soil averaged greater than 85%, as measured by High Pressure Liquid Chromotography.

Phase three consisted of a greenhouse study in which each of three wastes was applied to two soils. Results from chemical analyses indicate that waste constituents were degraded in soil during a 360 or 340 day interval. Increased mutagenic activity was exhibited in some soil and water extracts during this same interval. When compared on an equivalent volume basis, however, mutagenic potential of waste-amended soils was reduced over time and, in some cases, was reduced to a non-mutagenic level.

Wood-preserving bottom sediment was applied to barrel-sized lysimeters in the final project phase to compare results of soil-core and soil-pore liquid monitoring. Different types of compounds were detected in soil-core and soil pore liquid samples.

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Although the research described in this article has been funded wholly or in part by the U.S. Environmental Protection Agency under assistance agreement CR807701 to Texas A&M University, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

FOREWORD

EPA is charged by Congress to protect the Nation's land, air and water systems. Under a mandate of national environmental laws focused on air and water quality, solid waste management and the control of toxic substances, pesticides, noise, and radiation, the Agency strives to formulate and implement actions which lead to a compatible balance between human activities and the ability of natural systems to support and nurture life.

The Robert S. Kerr Environmental Research Laboratory is the Agency's center of expertise for investigation of the soil and subsurface environment. Personnel at the Laboratory are responsible for management of research programs to: (a) determine the fate, transport and transformation rates of pollutants in the soil, the unsaturated zone and the saturated zones of the subsurface environment; (b) define the processes to be used in characterizing the soil and subsurface environment as a receptor of pollutants; (c) develop techniques for predicting the effect of pollutants on ground water, soil and indigenous organisms; and (d) define and demonstrate the applicability and limitations of using natural processes, indigenous to the soil and subsurface environment, for the protection of this resource.

This project was initiated to determine the utility of short-term bioassays in monitoring the operation of land treatment systems. Results indicate that selected organic hazardous wastes can be treated in land treatment units and that bioassays can be used to monitor detoxification of organic hazardous waste constituents. This information should prove useful to those responsible for regulating, designing, and operating hazardous waste land treatment systems.

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ABSTRACT

A four phase study was conducted to evaluate the utility of short-term bioassays in monitoring the environmental impact of the land treatment of hazardous industrial wastes. In the waste characterization phase, the acute toxicity of ten wastes was evaluated in bioassays using *Bacillus subtilis*, *Salmonella typhimurium*, and haploid and diploid forms of *Aspergillus nidulans*. Based on the results of the acute toxicity study and a chemical characterization, three wastes were selected for an evaluation of chronic toxicity. The acid, base, and neutral fractions of each of the three wastes induced genetic damage in at least two of the three bioassays.

In the second phase, the additive effect of 2-nitrofluorene or benzo[a]pyrene to the soil was evaluated using both the prokaryotic point mutation assay and high performance liquid chromatography. The results from this portion of the study have been used to evaluate the efficiency of the blender and soxhlet extraction procedures, as well as the potential interactions between known mutagens and soil components. The results indicate that while greater quantities of hydrocarbons were extracted using the Soxhlet method, there was no appreciable difference in the mutagenicity of the extract using either procedure. In addition, when pure compounds were added to the soil, the extraction efficiency averaged greater than 85%, as measured by HPLC; while there was no statistical difference in the mutagenicity of the pure compound or the extract of the soil plus the compound.

Phase three consisted of a greenhouse study in which each of three wastes was applied to two soils. Soil, plant, and runoff samples were collected at various times over a 360 or 540 day interval. The results from chemical analysis indicate that waste constituents were degraded in soil; however, the bioassay results indicate that the degradative process may have increased the mutagenic potential of soil and water extracts, as well as converting indirect acting mutagens to direct acting compounds.

Barrel-sized lysimeters were used in the final phase of the project to compare soil-core and soil-pore liquid monitoring. A wood-preserving bottom sediment waste was applied to three lysimeters. Three additional lysimeters to which no waste was applied served as a control. Leachate and soil samples were collected prior to, 30, and 90 days after waste application. The results from this portion of the study indicate that if a land treatment facility is not properly managed, mutagenic constituents from land applied waste may migrate through the soil. The results from this research have demonstrated that hazardous industrial wastes may contain significant quantities of mutagenic materials. Soil incorporation of three wastes resulted in the transformation or degradation of mutagenic materials, although these reactions did not always reduce the mutagenic activity of soil extracts. In addition, for the waste, soil, and loading rate evaluated in the lysimeter study, attenuation of waste constituents was not sufficient to prevent mutagenic compounds from migrating to a depth of 90 cm. This research has demonstrated that short-term bioassays can be used to trace the environmental fate of mutagenic constituents in land applied hazardous industrial wastes.

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SECTION 1

INTRODUCTION

Industrial activities in the United States have undergone a rapid expansion over the past three decades concurrent with the development of numerous new chemicals. These activities have been responsible in part for a dramatic improvement in the quality of life while affecting a reduction in the frequency of infectious disease. An unavoidable by-product of our industrial growth has been the generation of large volumes of complex industrial wastes. Since 1950, the world production of organic chemicals has risen from 7 million tons to a projected 150 million tons by 1985 (U.S. Tarriff Commission, 1974). Presently, approximately 700 new chemicals are introduced into commerce each year, in addition to the more than one-half million chemicals already in use (National Cancer Institute, 1976). For the majority of these chemicals, there is little information available pertaining to their toxicological and environmental aspects (Fishbein, 1979).

This lack of sufficient information and the fact that the majority of chemical carcinogens are considered to be products of our increasing agricultural and technological sophistication (Chemical and Engineering News, 1975) dictate the need to develop techniques for monitoring the disposal of hazardous chemicals. The EPA (1983) estimated that 150 million metric tons of hazardous waste are generated annually in the United States. Past disposal practices have generally included deep sea dumping, incineration, and landfilling. All of these methods have environmental or economical drawbacks which limit their utility. The alternative disposal method of land treatment, i.e., the incorporation of waste into the surface layer of soil resulting in the degradation or attenuation of hazardous waste constituents, is being used more frequently for the disposal of selected industrial wastes. EPA (1980) regulations state that a waste cannot be land applied unless the waste is rendered less or non-hazardous by chemical or biological reactions in the soil. In order for land treatment to be a viable method of disposal, techniques are needed for the monitoring of hazardous waste constituents and their metabolites. The use of a combined biological and chemical testing protocol may provide the most practical means of efficiently monitoring hazardous waste disposal. The use of chemical analysis alone fails to account for the interactions of the components of a complex mixture, the production of mutagenic metabolites via degradative pathways, and chemical reactions between non-toxic precursors that may result in the formation of mutagenic compounds. An appropriately selected bioassay should be capable of integrating these effects. The use of biological analysis alone, however, could fail to account for artifacts generated in the collection or extraction process. A combined testing protocol utilizes microbial bioassays to measure primary DNA damage and a chemical analysis to identify causitive agents in genotoxic samples. Although not all chemical mutagens are established carcinogens, most chemical carcinogens have been determined to be mutagenic in sub-mammalian systems (Stolz et al., 1974; Miller and Miller, 1974; Committee 17, 1974). The utility of microbial mutagenesis assays is further enhanced by recent evidence that a point mutation in a human cell can give the cell malignant properties (Santos et al., 1984). Thus, microbial bioassays may be utilized to evaluate the mutagenic activity of waste and environmental samples as an indication of their potential for inducing mutagenic damage in the human population. Mutagenic compounds in the environment affecting somatic cells may induce cell death, cancer, aging, and heart disease (Ames, 1979); while mutations in germ cells may result in birth defects, sterility, and abortions (Brusick, 1981) (Figure 1). The human genome is our most precious heritage (de Serres, 1979), and analytical techniques must be developed to reduce the transmission of mutagenic defects to future generations. A need exists to determine if mutagenic constituents of land treated industrial waste will migrate from the site of application to locations where the human population could be exposed to them.

The objectives of this research were to characterize the genotoxic constituents of three hazardous wastes, to monitor waste degradation in soil, and to determine the environmental fate of mutagenic waste constituents following land application. In order to meet these objectives and to develop a set of test protocols which can be used to monitor environmental contamination, this project was divided into four main phases. The waste characterization phase included an acute toxicity evaluation of ten wastes, a complete characterization of the mutagenic potential of seven subfractions of three selected wastes, and a chemical characterization of major organic constituents. In the second phase, direct and indirect acting mutagens were added to the soil in order to quantify extraction procedures and to determine the affect of soil components on the activity of mutagenic compounds.

Phase three consisted of a greenhouse study in which three wastes were applied at one loading rate to two soil types packed in boxes. Simulated rainfall was applied and runoff and soil samples were collected at various time intervals during a 360 or 540 day period. The results from this phase of the project were used to evaluate the affect of degradation on the mutagenic activity of waste amended soil and the potential for the removal of mutagens in runoff water. In the final phase of the study, one waste was applied to an undisturbed soil enclosed in lysimeters in order to monitor the movement of mutagens through soil. The information generated by this research should provide a sound basis for determining the utility of short-term bioassays to

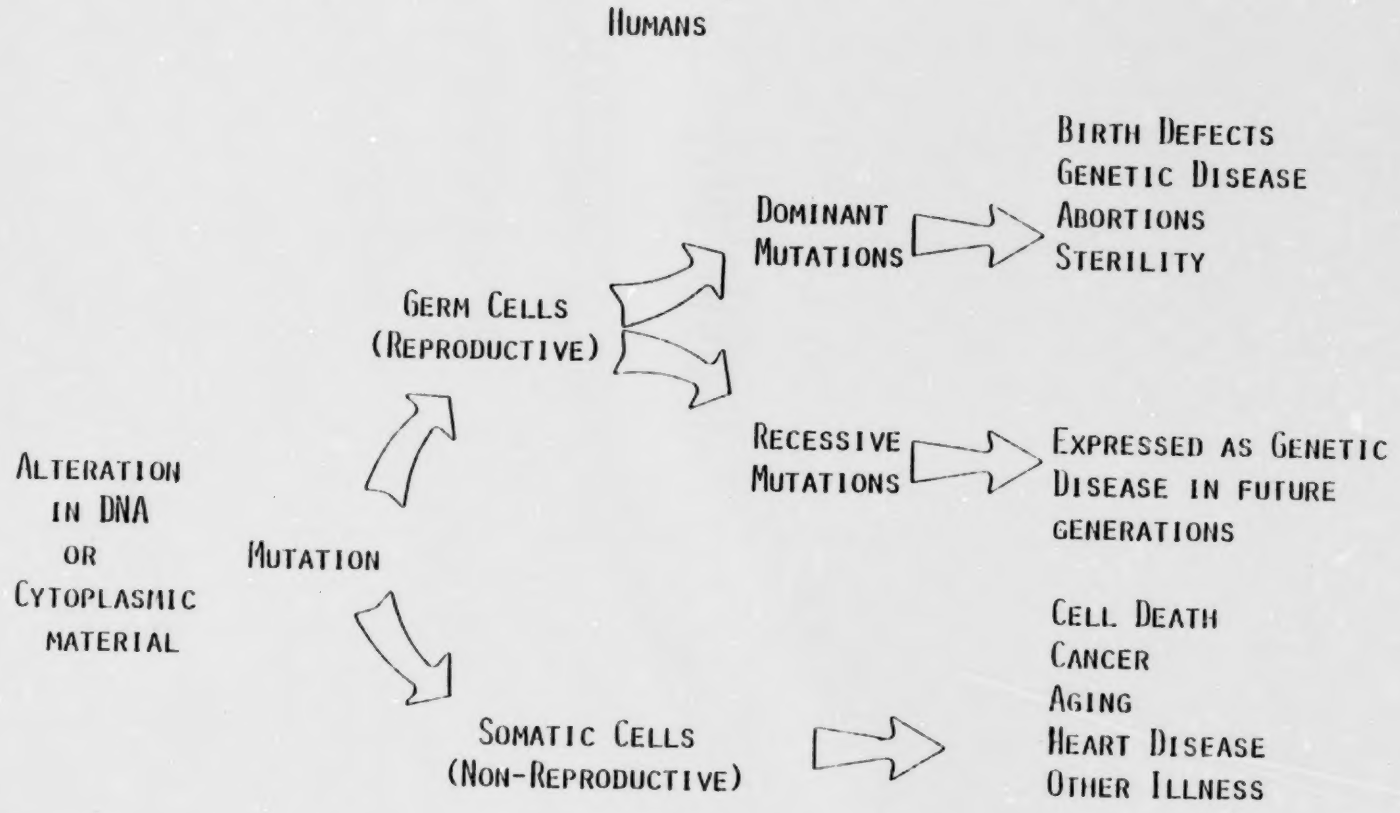
HUMANS
ALTERATION
IN DNA
OR
CYTOPLASMIC
MATERIAL
MUTATION
GERM CELLS
(REPRODUCTIVE)
SOMATIC CELLS
(NON-REPRODUCTIVE)
DOMINANT
MUTATIONS
RECESSIVE
MUTATIONS
BIRTH DEFECTS
GENETIC DISEASE
ABORTIONS
STERILITY
EXPRESSED AS GENETIC
DISEASE IN FUTURE
GENERATIONS
CELL DEATH
CANCER
AGING
HEART DISEASE
OTHER ILLNESS

Figure 1: Potential effects of environmental mutagens on human cells.

evaluate the environmental impact of the land treatment of hazardous industrial wastes.

SECTION 2

CONCLUSIONS

1. The battery of biological test systems employed in this research provided detection capabilities for a range of genotoxic damage and a variety of genotoxic compounds.

2. The acid, base, and neutral fractions of each of three hazardous wastes contained compounds capable of inducing DNA damage, point mutations, or chromosome damage.

3. An agricultural soil with no previous history of waste application will have an inherent level of mutagenic activity. The magnitude of this activity will be directly related to the past history of agricultural practices, including biocide applications, fertilization, and cultivation.

4. Through use of the soxhlet technique, significantly greater quantities of hydrocarbons were extracted from the waste than were extracted using the blender technique. However, the composition of the extracted hydrocarbons appeared to be similar using either technique, as there was no appreciable difference in the mutagenicity of the residues.

5. The extraction efficiency for 2-nitrofluorene and benzo(a)pyrene from soil using the blender technique and measured by HPLC averaged greater than 85%, while the mutagenic activities of the pure compound and the pure compound extracted from soil were within one standard deviation of each other.

6. The mutagenic potential of runoff water from soil amended with two of the three wastes was reduced 360 days after waste application. For the third waste, the mutagenic potential was increased, although the amount of extractable hydrocarbons in the runoff had decreased.

7. Degradation increased the mutagenic potential of the extractable hydrocarbons present in the acid and base fractions of all three waste amended soils. However, the mutagenic potential of equivalent volumes of waste amended soils was reduced and, in some cases, was reduced to a nonmutagenic level.

8. The wood-preserving waste collected for this study contained a limited number of highly mobile and highly mutagenic constituents that were identified by chemical and biological analysis. Different types of compounds were detected in soil core and soil pore liquid samples.

9. Chemical assisted biological analysis provided more accurate information for monitoring complex mixtures in the environment than either method alone.

SECTION 3

RECOMMENDATIONS

1. A battery of bioassays, capable of detecting point mutations, DNA repair damage, and chromosome damage should be used to define the genetic toxicity of a hazardous industrial waste.
2. The bioassay(s) used for environmental monitoring need only consist of the least complex test system(s) found to be sensitive to the type of genetic damage and the types of genotoxic compounds in the waste.
3. Both chemical and biological analysis should be used to monitor hazardous waste land treatment.
4. A field study would provide a more accurate representation of the potential of the three wastes used in the present study to be rendered less hazardous by soil incorporation.
5. A biological testing protocol used to monitor hazardous waste land treatment should include both chronic and acute toxicity bioassays.
6. Additional areas of research not evaluated in the present study which merit future investigation include the mutagenic potential of different wastes and the affect on detoxification of loading rate and fertilizer additions.

SECTION 4

BIOLOGICAL ANALYSIS

INTRODUCTION

Bioassay techniques measure the toxic effects of a substance by comparing its effect on a living organism to that of standard substances with known toxicological effects. A battery of short-term bioassays was used in this research to evaluate the genotoxic potential of various samples associated with land treatment of hazardous industrial waste. Through the use of a battery of bioassays to detect a range of genetic damage, environmental monitoring can be made both more economical and environmentally sound.

Genotoxic compounds in a hazardous waste should be monitored in order to control accidental exposure to mutagenic, carcinogenic, or teratogenic agents and to prevent the transmission of related genetic defects to future generations. Because no single test system has been identified which can detect all types of genetic damage and all genotoxic compounds, genetic toxicity can best be determined using a battery of biological test systems. The battery of test systems should be capable of detecting inhibition of DNA repair, gene mutations, and various types of chromosome damage.

The most common biological test systems employ microorganisms to detect point mutations in a haploid genome. Since the composition of DNA is essentially the same in all organisms, an agent which induces mutagenesis in a microorganism is also assumed to be capable of mutating mammalian DNA. While chemical mutagenesis is a relatively simple process involving the induction of heritable change in the genome, mutagenic events may constitute only the initial step of a multi-stage process in chemical carcinogenesis (Brusick, 1981). The eventual outcome of chemical carcinogenesis will be greatly influenced by enzyme activation and detoxification systems (Miller and Miller, 1974), as well as cocarcinogenic and promotional factors (Brusick, 1980). Therefore, not all microbial mutagens will also be carcinogens; although in several validation studies, 80 to 90% of carcinogens were detected as mutagens (McCann et al., 1980).

The most efficient battery for evaluating the genotoxic potential of environmental samples should be capable of detecting both a range of genetic damage and a range of genotoxic compounds. Each test system should include provisions for solvent and positive controls that will demonstrate the sensitivity of the test systems, the functioning of the metabolic activation system, and act as an internal control for the biological system. The following discussion concerns the standard protocols for the bioassays utilized in this research, as well as a comparison of the variability over time of each system, the types of damage and compounds detected, and the limitations of each system.

A. Differential DNA Repair

1. Test System Description--

Repair assays are used to detect primary DNA damage and evaluate differential growth inhibition of DNA repair proficient and deficient pairs of bacteria. Increased growth inhibition in a DNA repair deficient strain of bacteria implies that the agent being tested has reacted with cellular DNA to produce a repairable DNA lesion. Agents which produce DNA damage are likely to be mutagenic, carcinogenic, as well as effectors of chromosome aberrations (Kada, 1978). Numerous test systems are available which employ pairs of *E. coli* (Slater *et al.*, 1971), *B. subtilis* (Kada *et al.*, 1977; Tanooka, 1977; Felkner *et al.*, 1979), *P. mirabilis* (Adler *et al.*, 1976), and *S. typhimurium* (Ames *et al.*, 1975). These test systems offer the advantage of being rapid, simple, and inexpensive. In addition, since these tests are capable of detecting DNA damage at any locus and by any mechanism, they are capable of detecting many compounds not detected by point mutation assays (Kada, 1980; Rosenkranz and Poirier, 1979). Limitations of DNA repair assays in bacteria include the minimal response from many compounds that are poorly soluble in water, the lack of response from large molecules due to the impermeability of the cell wall, and an inability to interpret borderline responses from the spot test (Leifer *et al.*, 1981). The following modifications to the standard protocol were used to enhance the sensitivity of the DNA repair assay.

2. Test system protocol: DNA repair--

To test for lethal DNA-damage, five different strains of *B. subtilis* deficient in different recombination (Rec$^-$) and/or excision (Exc$^-$) repair were employed. These included the Rec$^-$ strains *recA8*, *recE4*, and *mc-1*; Exc$^-$ strain *hcr-9*; and Rec$^-$, Exc$^-$ strain *fh2006-7*. These strains are all isogenic with *B. subtilis* strain *168* which has all repair intact (Table 1). The DNA-repair assay was performed using the procedure of Felkner (1977) on strains Dr. I. C. Felkner kindly supplied. Metabolic activation was incorporated into the spot test by combining 0.5 ml of the test chemical with 0.5 ml of S-9 mix. The S-9 mix is composed of enzymes obtained from the supernatant of homogenized rat liver which was centrifuged at 9,000 x g, and a NADPH generating system. This mix, when incorporated into the bioassay, simulates the in vivo biotransformation of the test material (Muller

TABLE 1. STRAINS OF BACILLUS SUBTILIS USED FOR MUTAGEN TESTING

Strain	Genotype[1]	Source
168 wt	Wild-type	Dr. I. C. Felkner
rec E4	trp, thr, Rec$^-$	
rec A8	trp, thr, Rec$^-$	
rec B2	trp, thr,. Rec$^-$	
hcr-9	trp, thr, Exc$^-$	
fh2006.7	trp, thy, Rec$^-$, Exc$^-$	
mc-1	trp, Rec$^-$	
TKJ5211	his, meth, Exc$^-$	
TKJ6321	his, meth, polA151, spp-1, Exc^{-1}	

1. Auxotrophic mutations: trp (tryptophane), thr (threonine), thy (thymine), his (histidine), meth (methionine).

Repair Deletions: Rec$^-$ (recombination), Exc$^-$ (excision), polA151 (polymerase).

Additional Mutations: spp-1 (spore-repair deficiency marker).

et al., 1980). Aroclor 1254 induced rat liver was obtained from Litton Bionetics (Charleston, S.C.) and prepared according to the procedure of Ames et al. (1975). The chemical and S-9 mixture were incubated with shaking for 30 min at 37°C. Inocula from overnight cultures were streaked radially on a nutrient agar plate to either a centered sensitivity disk containing 100 µl of the test sample or a well in the agar plate containing 200 µl of the chemical S-9 mixture. The plates were incubated overnight at 37°C, and the distance of growth inhibition was measured from the periphery of the disk or well. Dimethyl sulfoxide (DMSO) was used as a negative control. Mitomycin C (Sigma), methylmethane sulphonate (Aldrich), and sensitivity to ultraviolet light were used as appropriate positive controls.

Fractional survival (N/N_o) was evaluated for the most sensitive strains and for strain 168 using the procedure of Felkner et al. (1979). Cells were inoculated into Difco brain-heart infusion broth (BHI) and incubated for approximately 16 hours until an $O.D._{540}$ of 0.3 was reached. The cells were then diluted with fresh BHI to an $O.D._{540}$ of 0.1 (approximately 1.2×10^8 cells/ml) and serially diluted with Spizizen's Minimal Salts (Spizizen, 1958). To determine fractional survival, 100 µl of the bacterial suspension at a minimum of three cell concentrations was added to 2.5 ml of nutrient top agar in a dry bath at 47°C. To this mixture, either 100 µl of the test sample or 200 µl of the sample S-9 mixture was added at 37°C and plated onto nutrient agar in duplicate. Plates were incubated for 24 hours at 37°C, and the surviving fraction was determined by dividing the colony count on the treated plate (N) by the colony count for the solvent control (N_o). The results shown are the average of two independent experiments with duplicate platings at each of three cell concentrations.

3. Test system results--

The results presented in Table 2 indicate that water soluble mutagens such as mitomycin C (MitC) are readily detected using the spot test, while compounds such as benzo(a)pyrene [B(a)p] or 2-nitrofluorene (2-NF) which require metabolic activation or are sparingly soluble in water give no response. An evaluation of fractional survival in two repair-deficient strains recE4 and fh2006-7 (Table 3) indicates that 2-NF does produce increased lethal damage in the repair-deficient strains. The use of a plate-incorporation assay to evaluate survival apparently provided a greater opportunity for cellular exposure to 2-NF, and thus the positive response was obtained. Both MitC and MMS continued to induce a positive response in the plate incorporation assay, and results from this assay were more easily transformed into a dose-response curve than for the spot test (Table 3).

For the indirect-acting mutagens B(a)p and 2-AA, no response was obtained in the spot test with or without activation, or in the survival assay without activation (Table 1 and 4). However, when metabolic

TABLE 2. COMPARISON OF LETHAL EFFECT OF VARIOUS SAMPLES ON DNA REPAIR DEFICIENT AND PROFICIENT STRAINS OF B. SUBTILIS

Sample	S-9	Dose/Plate (µg)	Inhibition Radius (mm)						
			168 wt RP[1]	recE4	fh2006.7	recA8	mc-1	hcr.9	Response[2]
				repair deficient					
Mit C	−	20	4.3	12.4	7.2	11.5	10.2	15.1	+++
MMS	−	2	6.8	13.0	15.9	15.6	17.8	12.1	+++
2NF	−	500	0	0	0	0	0	0	−
B(a)p	+	50	0	0	0	0	0	0	−
	−	50	0	0	0	0	0	0	−
2AA	+	100	0	0	0	0	0	0	−
	−	100	0	0	0	0	0	0	−
DMSO	−	100 µl	0	0	0	0	0	0	−
	+	100 µl	0	0	0	0	0	0	−

1- Repair Proficient

2- Response: (zone of inhibition in repair deficient strain-zone of inhibition in repair proficient strain) <3 = −; 3,4 = +; 5-7 = ++; >7 = +++.

TABLE 3. SURVIVAL OF WILD-TYPE AND REPAIR-DEFICIENT STRAINS OF *B. SUBTILIS* EXPOSED TO VARYING CONCENTRATIONS OF DNA DAMAGING CHEMICALS

Sample	Dose/Plate (μg)	Fractional Survival (N/N_o)		
		168 wt	recE4	fh2006.7
Methylmethane sulfonate	20	0.27	0.0007	0.0005
	2	0.83	0.60	0.26
	0.2	0.92	0.43	0.70
	0.02	1.04	0.69	0.44
Mitomycin C	2	0.72	0.03	0.20
	1	0.91	0.13	9.14
	0.1	0.97	0.62	0.62
	0.01	0.91	0.77	0.57
2-nitrofluorene	1,000	0.95	0.47	0.34
	500	0.82	0.60	0.52
	250	1.04	0.53	0.42
	25	0.99	0.51	0.39
	2.5	1.00	0.90	0.45

TABLE 4. SURVIVAL OF WILD-TYPE AND REPAIR-DEFICIENT STRAINS OF B. SUBTILIS EXPOSED TO VARYING CONCENTRATIONS OF 2-AMINOANTHRACENE AND BENZO(A)PYRENE WITH AND WITHOUT METABOLIC ACTIVATION

Sample	Dose/Plate (μg)	Fractional Survival N/N_0					
		168 wt		recE4		fh2006.7	
		+ S-9	- S-9	+ S-9	- S.9	+ S-9	- S.9
Benzo(a)pyrene	10	0.70	-	0.24	-	0.38	-
	5	0.91	0.71	0.39	0.46	0.43	0.75
	0.5	0.86	0.60	0.33	0.73	0.48	0.83
	0.05	0.81	0.65	0.29	0.63	0.57	0.93
	0.005	0.85	0.82	0.65	0.69	0.47	0.76
2-aminoanthracene	10	0.52	0.73	0.24	0.54	0.37	0.43
	1	0.47	0.81	0.35	0.86	0.38	0.51
	0.1	0.57	0.85	0.49	0.61	0.49	0.47
	0.01	0.86	0.83	0.42	0.76	0.67	0.61
	0.001	0.102	0.94	0.76	0.94	0.71	0.55

activation was incorporated into the survival assay by pre-incubation of the chemical S-9, both B(a)p and 2-AA induced increased lethal damage in DNA repair deficient strains (Table 4, Figures 2 and 3). After exposure to the 2AA:S-9 mixture at the highest dose level, the survival ratio of _recE4:168_ was 0.46. This would be considered a positive response, and at lower dose levels this effect was reduced. The survival ratio for _recE4:168_ at 5 and 0.5 µg/plate exposure to B(a)p:S-9 mixture was 0.43 and 0.33, respectively. These results indicated that while both 2-AA and B(a)p induced increased lethal damage in DNA-repair deficient bacteria after metabolic activation, the DNA-damaging activity of B(a)p appeared to be greater.

This protocol increased the utility of a plate-incorporation assay that employed several different DNA repair-deficient strains of bacteria with metabolic activation. In the _Salmonella_/microsome assay, the frequency of induced mutations per nanomole was found to be four times greater for 2AA than for B(a)p (McCann _et al_., 1975). This may have been due in part to increased lethal damage induced by B(a)p in the excision repair deficient strains used for mutagenesis testing. The addition of a DNA-repair assay to a battery of test systems for detecting potential carcinogens should enhance the sensitivity of the battery towards compounds such as MitC which cause DNA damage at low dose levels. By utilizing a plate incorporation assay with metabolic activation, the sensitivity of the DNA repair assay can be enhanced toward compounds which are indirect-acting or sparingly soluble in water. This slight modification to the standard procedure for measuring fractional survival in _B. subtilis_ produces an increased sensitivity towards certain genotoxic compounds and should increase its utility as part of a battery of biological test systems. The DNA repair assay using _B. subtilis_ offers the additional advantages of increased permeability of the gram positive organisms and the use of multiple strains of repair deficient organisms which can detect inhibition of different types of DNA repair (Tanooka, 1977).

B. Point Mutations: Prokaryote

1. Test system description--

Microbial mutagenesis assays measure primary DNA damage by detecting small changes in the nucleotide sequence of DNA. The more common reverse mutation assays detect changes (point mutations) by measuring bacterial growth in a media free of a specific substrate which the bacteria require for growth. Increased colony formation (or growth) following exposure to a specific agent implies that the agent has induced reverse mutations. Damage to DNA by environmental mutagens may contribute to human cancer, genetic defects, aging, and heart disease (Ames, 1979). However, since reverse mutations are highly specific in the type of required DNA interaction, a compound that does not increase the frequency of reverse mutations may still induce other types of genetic effects (Anderson and Longstaff, 1981). For this reason, a more

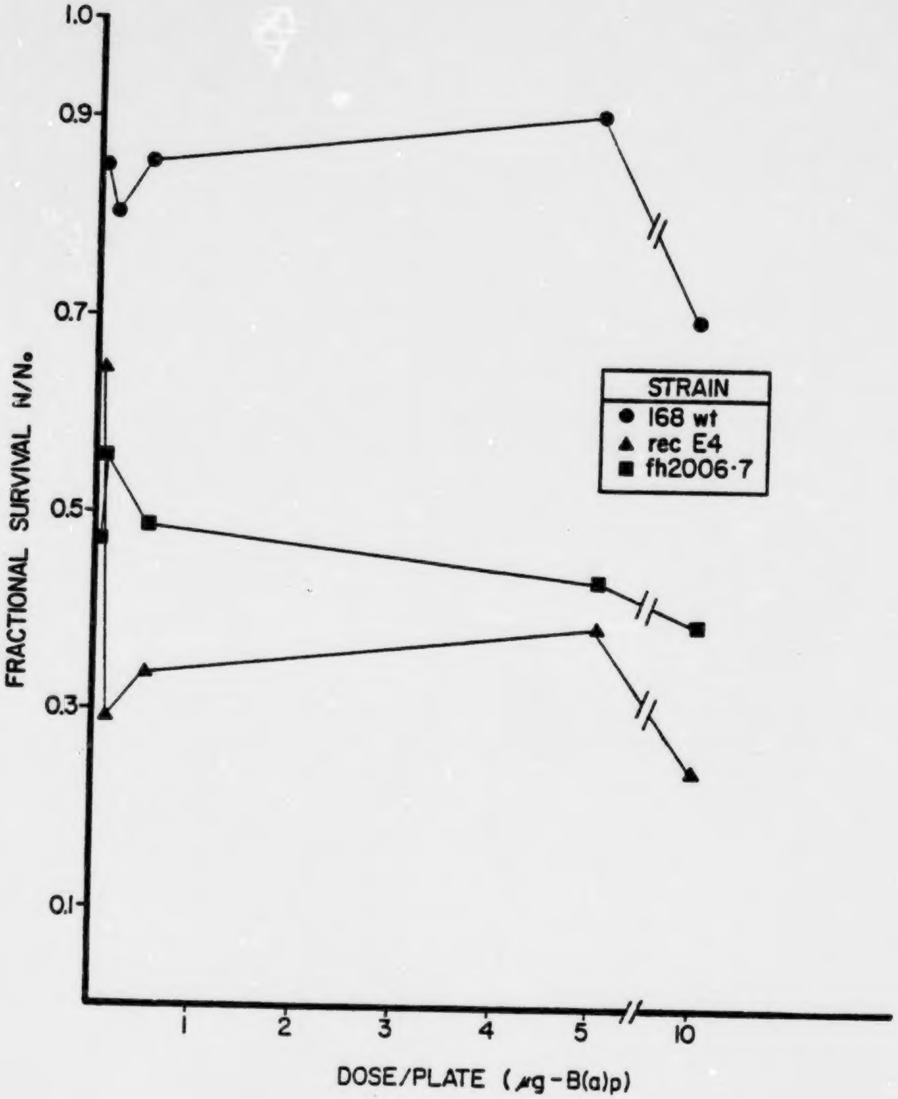

Figure 2. Lethal effect of increasing doses of benzo(a)pyrene on repair proficient (168 wt) and deficient strains of B.subtilis with metabolic activation.

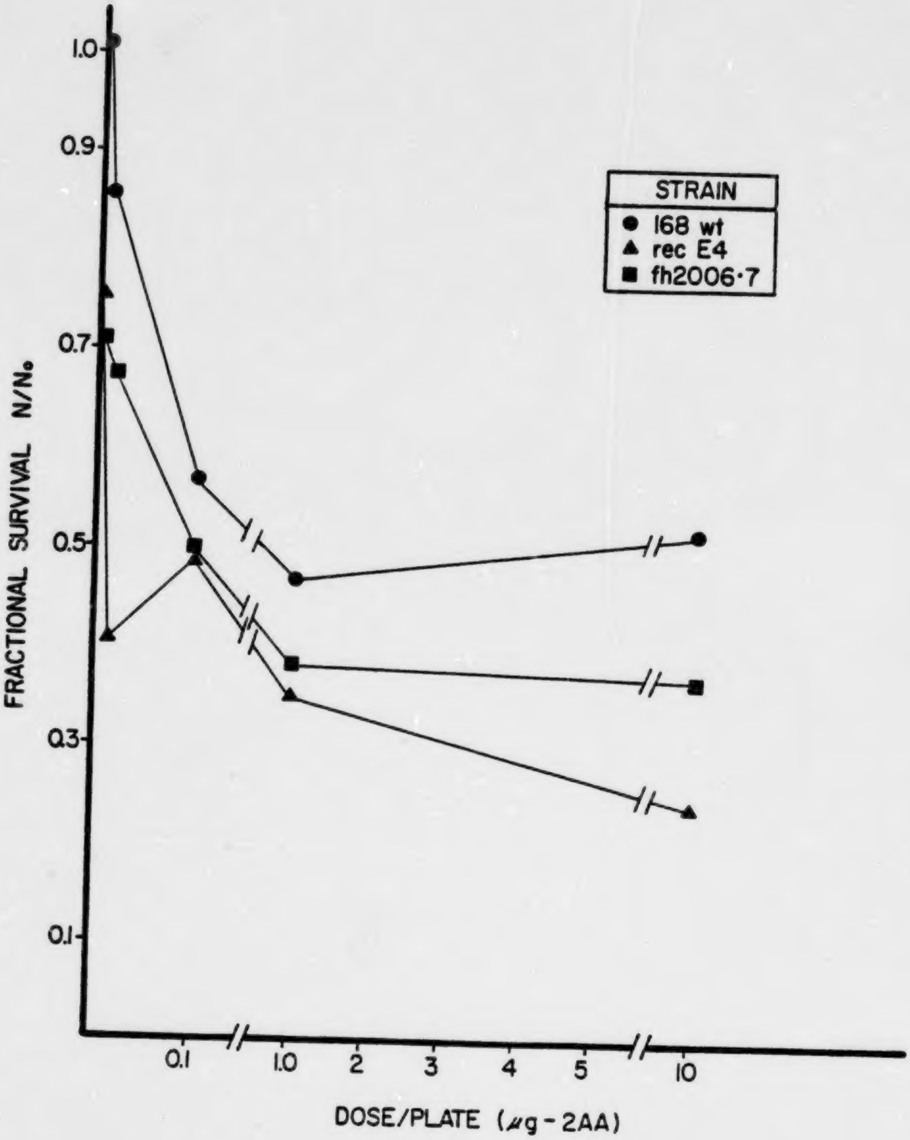

Figure 3. Lethal effect of increasing doses of 2-amino-anthracene on repair proficient (168 wt) and deficient strains of B.subtilis with metabolic activation.

accurate risk assessment is obtained when a battery of bioassays are used to detect a range of genetic damage.

The most widely validated microbial mutagenesis assay is the Salmonella/microsome assay developed by Ames et al. (1973). Using the Salmonella assay, test data has been published on more than 5,000 chemicals (Environmental Mutagen Information Center Index, 1982) and several validation studies have been conducted (McCann et al., 1975; Purchase et al., 1976; Sugimura et al., 1976; Bartsch et al., 1980; De Flora, 1981). The correlation between carcinogenicity and mutagenicity using this test is currently estimated to be about 83% (McCann and Ames, 1981). Categories of carcinogens which are poorly detected by the Salmonella assay include azonapthols, carbamyls, and thiocarbamyls; phenyls; benzodioxoles; polychlorinated aromatics, cyclics, and aliphatics; steroids; antimetabolities; and symetrical hydrazines (Rinkus and Legator, 1979).

In the present study, two bacterial assays have been utilized to evaluate the capacity of various samples to induce point mutations. The B. subtilis reverse mutation assay, as described by Tanooka (1977), was used with selected waste fractions to supplement the Salmonella assay. The B. subtilis assay has been found to be sensitive to many compounds poorly detected by the Salmonella assay (Felkner, 1981) and offers the advantage of being a gram positive organism that is more permeable to certain compounds (Tanooka, 1977). The following discussion will include the protocols used for the two prokaryotic mutagenesis assays and the results obtained using various diagnostic mutagens.

2. Test system protocol: prokaryote mutagenesis--

The Salmonella/microsome assay as described by Ames et al. (1975) was used to measure the ability of a sample to revert strains of bacteria to histidine prototrophy. A description of the S. typhimurium strains that were provided by Dr. B. N. Ames (University of California, Berkeley, CA) is given in Table 5. The procedures used were essentially the same as Ames et al. (1975). Overnight cultures were grown from individual colonies picked from nutrient agar master plates and inoculated into Oxoid Nutrient Broth No.2 (KC Biological, Lenexa, KS). Cultures were incubated for 16 hr at 37°C. All strains were checked quarterly or when new master plates were made for nutritional markers for the following characteristics: the deep rough character, the presence of the R factor, and sensitivity to ultraviolet light. Top agar and petri plates were prepared as described by Ames et al. (1975). Samples were tested with strains TA98 and TA100 in two independent experiments at five dose levels on duplicate plates with and without metabolic activation (0.3 ml rat liver/1.0 ml S9 mixture; 0.5 ml S9 mixture/plate). Aroclor 1254-induced rat liver was obtained from Litton Bionetics (Charleston, S.C.). Positive controls, as well as solvent and sterility controls, were run on each test date. The positive control used to verify functioning of the metabolic activation

TABLE 5. CHARACTERISTICS OF SALMONELLA STRAINS USED FOR MUTAGENESIS TESTING

Strain	Histidine Mutation	Additional Mutations[1]	SMF[2]	IMF[3]	IMF + S9[4]
TA1535	his G46	LPS; Exc$^-$	39	2774	440
TA100	his G46	LPS; Exc$^-_{-1}$R$^+$	120	2282	1833
TA1538	his D3052	LPS; Exc$_{-1}$	16	2158	870
TA98	his D3052	LPS; Exc$_-^{1}$ R$^+$	26	2421	1594
TA1537	his C3076	LPS; Exc$^-$	9	160	371

1- LPS$^-$ - lipopolysaccharide cell wall.
Exc$^-$ - excision repair deficient.
R$^+$ - plasmid pKM101

2- Historical spontaneous mutation frequency for lab with DMSO, without S9.

3- Induced mutation frequency; TA1535, TA100-MNNG: TA98, TA1538-2NF; TA1537-9AA

4- Induced mutation frequency 2-acetylaminofluorene with activation.

system for all strains was 10 μg 2-aminoanthracene (Sigma, St. Louis, MO); while direct acting positive controls for TA98 and TA1538 were 25 μg 2-nitrofluorene, for TA100 and TA1535 2 μg N-methyl-N'-nitro-N-nitrosoguanidine, for TA97 and TA1537 10 μg 9-aminoacridine, and 1 μg mitomycin C for TA102. Dimethyl sulfoxide (DMSO) was used as a negative control.

A limited number of samples were also analyzed using the *Bacillus subtilis* reverse mutation assay (Tanooka, 1977; Felkner *et al.*, 1979). The two *B. subtilis* mutants were supplied by Dr. I. C. Felkner (Clements Assoc., Washington, D.C.) and are described in Table 1. These strains may be used to detect either reverse mutation to His^+ or Met^+ or a forward mutation to prototrophy as His^+, Met^+. In the present study, only the reverse mutation assay was utilized.

The methods employed were basically the same as described by Tanooka (1977). Cells of TKJ5211 or TKJ6321 were grown overnight at 37°C with shaking in Oxoid Nutrient Broth No. 2. The cells were then harvested, resuspended in an equal volume of glucose minimal medium, and starved for 1 h at 37°C with shaking. To detect the His^+ mutation, 0.2 ml of culture was plated with 2.5 ml soft agar on a suppplemented minimal media (Tanooka, 1977). Plates were incubated for 72 hrs at 37°C.

Statistical Analysis--Mutagenicity in the *Salmonella*/microsome assay was determined using the modified two-fold rule (Chu *et al.*, 1981). To determine the mutagenicity of a sample, the mutagenic activity ratio (MAR) is first calculated (Commoner, 1976) at the four highest non-toxic dose levels. The mutagenic activity ratio is defined as $(E-C)/C_{Ave}$, where E is the experimental number of revertant colonies (average of four plates); C is the number of revertant colonies on the control plates (average of eight plates) obtained on the same day with the same strain and microsome preparation; and C_{Ave} is the historical average of revertant colonies on control plates obtained the year the test was run with the same strain and microsome preparation. A sample was considered mutagenic if the MAR was greater than 2.0 for at least two consecutive dose levels or in the last non-toxic dose level. In addition, at least two of these consecutive doses must possess a dose-response relationship showing an increase in the number of mutant colonies at increasing dose levels. Chu *et al.* (1981) calculated that when using the modified two-fold rule to determine the mutagenic potential of a sample, false positives and false negatives will occur at a rate of 4.1% and 1.8%, respectively. In an evaluation of seven different methods for statistical analysis of data from microbial mutagenicity assays, Chu *et al.* (1981) determined that the modified two-fold rule was one of three analytical methods that gave an acceptable rate of false positives and negatives. All raw data from the *Salmonella* assay was first entered into a TRS-80 computer using a program that calculated the means and standard deviations. This information is provided in Appendix C. Selected data was then entered into a second TRS-80 program to calculate the mutagenic activity ratios. A printout of the programs utilized to process data is provided in Appendix D.

3. Test system results: prokaryotic mutagenesis--

Prokaryotic mutagenesis assays using both S. typhimurium and B. subtilis were employed in this research. However, the vast majority of the work was conducted using the Salmonella/microsome assay of Ames et al. (1975). Recent modifications to the standard protocol (Maron and Ames, 1983) and the addition of two new tester strains (Levin et al., 1982a; Levin et al., 1982b) have enhanced the sensitivity of this bioassay. Brusick (1983) reviewed the sensitivity (mutagenic carcinogens/total carcinogens), specificity (nonmutagenic noncarcinogens/total noncarcinogens), and predictive value (mutagenic carcinogens/total mutagens) of the Salmonella assay as reported in the literature. Based on studies which reviewed at least 100 chemicals, Brusick (1983) reports that the sensitivity ranges from 54 to 93%, the specificity ranges from 77 to 100%, and the predictive value ranges from 87 to 100%. Overall, Brusick (1983) reports that the true correlation coefficient is about 80% when compared to tumor responses in mice and rats. Groups of chemicals which are poorly detected in bacterial mutagenesis assays include antimetabolites, azo compounds, carbamyls and thiocarbamyls, halogenated compounds, steroids, cross-linking agents, inorganic compounds, and promotors (Brusick, 1983). Most of these groups can be detected by using microbial assays which employ DNA repair deficient bacteria (Shiau et al., 1980; Kanematsu et al., 1980) or eukaryotic organisms (Scott et al., 1982; Kafer et al., 1982). Thus, when employed as part of a battery of biological test systems, the Salmonella/microsome assay can provide a highly efficient tool for monitoring genotoxic chemicals in the environment.

The final interpretation of the results from the Salmonella/ microsome assay may be influenced by a variety of factors including the selection of solvents, the size of the inoculum, the presence or absence of metabolic activation, and the presence of histidine containing substances. Organic solvents are used in biological testing in order to dissolve compounds which are sparingly soluble in water and may affect the results of the bioassay due to the solvents' toxicity or interractions with cellular membranes and proteins (Abbondandolo et al., 1980). Organic solvents which are compatible with the Salmonella test include dimethyl sulfoxide, glycerol formal, dimethyl formamide, formamide, acetonitrile, 95% ethanol, acetone, ethylene glycol dimethyl ether, 1-methyl-2-pyrrolidinone, p-dioxane, tetrahydrofurfuryl alcohol, and tetrahydrofuran (Maron et al., 1981). Abbondandolo et al. (1980) observed that ethylene glycol, 2-methoxyethanol, and methanol were also compatible with mutagenesis test systems. While these 15 solvents provide a useful array of characteristics, certain fractions of a hazardous waste may contain substances which are insoluble in any of these solvents. The data provided in Table 6 indicate that a 1:10 mixture of dichloromethane in dimethyl sulfoxide may be used to increase solubility without significantly affecting the results. The use of such a combined solvent system can, however, affect the results of the *Bacillus* and *Aspergillus* assays (data not shown), and its use should be restricted to preliminary testing in *Salmonella*.

The standard procedures for the *Salmonella* assay (Ames *et al*., 1975) recommend the addition of 0.1 ml of an overnight (16 h) culture, or approximately 1 x 10^8 bacteria to 2.0 ml of top agar. In the revised methods, Maron and Ames (1983) suggest reducing the incubation period to 10 h in order to increase the viability of the cells plated. Matney (1981) and Green and Muriel (1976) state that the number of spontaneous revertants per plate is completely independent of the initial inoculum size within the range of 10^5 to 10^8 cells per plate. As for the effect of inoculum size on mutagen yield, both Belser *et al*. (1981) and Salmeen and Durison (1981) found no appreciable difference in the yield of revertants induced by 2-nitrofluorene at plating densities ranging from 1 x 10^4 to 1 x 10^7, although increasing the cell density to 1 x 10^8 did produce a 2-fold increase in revertant yield (Salmeen and Durison, 1981). MacPhee and Pallister (1983) predicted that inoculum size would have a significant affect on the results when testing a mixture of toxic and mutagenic compounds. They observed that the sensitivity of the assay was increased by decreasing the inoculum size to approximately 1 x 10^6 cells per plate. Barber *et al*. (1983) also observed a relationship between growth and reversion frequency in the *Salmonella* assay. They found that the final reversion frequency will be dependent on the L-histidine concentration in the top agar, and that no reversion takes place during the first four hours after the bacteria are plated (Barber *et al*., 1983). The fact that no reversion takes place during the first four hours after plating may explain the limited sensitivity of the plate incorporation assay to volatile compounds (Barber *et al*., 1983).

Additional factors may also affect the reversion frequency in the *Salmonella* assay. Peak *et al*. (1983) demonstrated that the liver-microsome S9 enzyme produces a highly significant increase in the spontaneous reversion frequency. This data is consistent with that of the present study (Table 7 and 8) which indicate that the rat-liver S9 extract alone increases the number of revertant colonies on control plates.

Trace quantities of histidine which may be present in biological material can also affect the results of the *Salmonella*/microsome assay (Aeschbacher *et al*., 1983). This affect may be especially important in the analysis of soil extracts. Aeschbacher *et al*. (1983) recommend using a solvent or sorbent extraction to eliminate interference from histidine. In the present study, XAD resins and dichloromethane were used to extract water and soil samples,

TABLE 6. EFFECT OF A 1:10 $MeCl_2$:DMSO* SOLUTION ON THE SPONTANEOUS REVERSION AND INDUCED MUTATION FREQUENCY IN _S. TYPHIMURIUM_

Strain	Sample	Revertants/Plate	
		DMSO	$MeCl_2$:DMSO
TA98	-	21	24
	2-nitrofluorene (25 µg)	3008	2485
	2-aminoanthracine (10 µg)	2007	1949
TA100	-	96	91
TA1535	-	19	21
TA1538	-	13	11
TA1537	-	10	9

* Dichloromethane:dimethylsulfoxide.

TABLE 7. RESPONSE OBTAINED IN PROKARYOTIC MUTAGENESIS ASSAYS USING S. TYPHIMURIUM OR B. SUBTILIS WITH NEGATIVE AND POSITIVE CONTROLS

Chemical	S-9	Dose/Plate (µg)	TA1535	TA1538	TA98	TA100	TKJ5211	TKJ6321
			Total Revertants Mean + SD					
None	-	0	36 ± 9	18 ± 8	30 ± 8	127 ± 24	25 ± 9	103 ± 14
	+	0	22 ± 4	NT	40 ± 15	126 ± 36	NT	NT
DMSO	-	100 µl	39 ± 10	16 ± 8	26 ± 6	120 ± 38	31 ± 8	131 ± 40
	+	100 µl	22 ± 6	35 ± 9	37 ± 10	122 ± 27	21 ± 6	112 ± 45
2-NF	-	250	NT	1,765 ± 572	2,421 ± 423	NT	417 ± 227	438 ± 205
		25	NT	2,158 ± 749	2,197 ± 445	NT	93 ± 49	203 ± 78
		2.5	NT	850 ± 253	632 ± 205	NT	NT	NT
MNNG	-	4	2,774 ± 136	NT	NT	1,743 ± 927	2,292**	1,519**
		2	2,547 ± 124	NT	NT	2,282 ± 404	39**	118**
		0.2	36 ± 10	NT	NT	452 ± 455	26**	109**
MMS	-	20	494 ± 457	NT	NT	1,296 ± 118	393 ± 281	261**
		2	268 ± 392	NT	NT	1,826 ± 415	54 ± 6.4	133**
		0.2	222 ± 212	NT	NT	555 ± 294	29 ± 11	108**
B(a)p	-	50	31 ± 4	22 ± 11	28 ± 7	131 ± 21	21 ± 5	124**
	+	500	49 ± 5	188 ± 15	507 ± 99	778 ± 247	329 ± 96	996 ± 175
		50	54 ± 6	156 ± 30	524 ± 137	889 ± 178	380 ± 33	371 ± 34
		5	50 ± 10	124 ± 22	609 ± 152	707 ± 130	149 ± 130	91 ± 26
		.5	32 ± 4	52 ± 9	86 ± 41	197 ± 31	26 ± 0.7	NT
2AA	-	10	54 ± 8	33 ± 8	37 ± 13	146 ± 37	60**	131 ± 3
	+	100	440 ± 188	NT	1,122 ± 469	868 ± 133	341**	608 ± 81
		10	346 ± 94	870 ± 343	1,548 ± 444	1,833 ± 554	774**	718 ± 274
		1	44 ± 13	NT	581 ± 319	671 ± 446	196**	253 ± 46

* Negative controls: none-no additions to top agar; DMSO-100 µl Dimethylsulfoxide; Postive controls: 2-NF-2-Nitrofluorene; MNNG-N-methyl-N'-nitro-N-nitrosoguanidine; MMS-methyl methane sulfonate; B(a)P- benzo(a)pyrene; 2AA-2-aminoanthracene; NT = not tested.

** Represent one experiment with duplicate plates, standard deviation not provided.

TABLE 8. VARIABILITY OF S. TYPHIMURIUM STRAINS TA98 AND TA100 WITH NEGATIVE AND POSITIVE CONTROLS FOR EACH YEAR OF THE PROJECT

Strain	Sample[1]	S-9	Dose/Plate	Year[2] 1981 (N)	1981 Mean ± SD	1982 (N)	1982 Mean ± SD	1983 (N)	1983 Mean ± SD
TA98	None	-	-	(NT)	-	(246)	29 ± 8	(217)	30 ± 8
		+	-	(NT)	-	(94)	38 ± 10	(123)	42 ± 15
	DMSO	-	100 µl	(72)	20 ± 4	(529)	25 ± 6	(440)	29 ± 7
		+		(105)	28 ± 10	(449)	34 ± 10	(448)	42 ± 10
	$MeCl_2$	-	10 µl	(NT)	-	(80)	33 ± 12	(NT)	-
		+		(NT)	-	(60)	37 ± 12	(NT)	-
	2NF	-	25 µg	(34)	1,760 ± 424	(112)	2,257 ± 594	(93)	1,943 ± 315
	B(a)P	-	50 µg	(NT)	-	(8)	27 ± 10	(6)	28 ± 9
		+		(NT)	-	(10)	501 ± 116	(6)	731 ± 88
	2AA	-	10 µg	(37)	24 ± 8	(123)	37 ± 16	(96)	41 ± 12
		+		(42)	2,142 ± 15	(126)	1,878 ± 431	(88)	1,751 ± 392
TA100	None	-	-	(NT)	-	(144)	119 ± 22	(197)	132 ± 25
		+	-	(NT)	-	(88)	123 ± 27	(135)	128 ± 42
	DMSO	-	100 µl	(56)	87 ± 11	(416)	113 ± 26	(386)	132 ± 55
		+		(91)	112 ± 27	(374)	114 ± 28	(373)	132 ± 26
	$MeCl_2$	-	10 µl	(NT)	-	(83)	116 ± 29	(NT)	-
		+		(NT)	-	(55)	114 ± 27	(NT)	-
	MNNG	-	2 µg	(28)	2,374 ± 602	(106)	2,321 ± 528	(78)	2,197 ± 549
	MMS	-	2 µg	(NT)	-	(8)	1,854 ± 254	(NT)	-
	B(a)P	-	50 µg	(NT)	-	(6)	124 ± 24	(8)	137 ± 19
		+		(NT)	-	(6)	603 ± 75	(8)	1,104 ± 256
	2AA	-	10 µg	(22)	109 ± 24	(113)	142 ± 39	(91)	160 ± 38
		+		(35)	2,054 ± 667	(110)	1,799 ± 580	(85)	1,787 ± 473

1. Negative controls: none = 3.0 ml top agar only; DMSO = dimethylsulfoxide; $MeCl_2$ = dichloromethane, 1:10 in DMSO; Positive controls: 2NF = 2-nitrofluorene; B(a)P = Benzo(a)pyrene; 2AA = 2-aminoanthracene; MNNG = N-methyl-N'-nitro-N-nitrosoguanidine; MMS = methyl methanesulfonate.

2. Year: 1981 - includes data from 6/1/81 through 12/31/81; 1982 includes data from 1/1/82 through 12/31/82 and 1983 includes data from 1/1/83 through 9/31/83; (N) = number of observations; NT = not tested.

respectively. It is anticipated that these procedures should eliminate any histidine in water and soil samples. Additional factors which may affect the results of mutagenicity tests include the method of media preparation and amount of medium per plate (Friederich et al., 1982), the inducer used for the liver enzymes (Venitt, 1980), and the presence or absence of visible light (MacPhee and Imray, 1974). Belser et al. (1981) reported that the greatest source of variability is nonuniformity of the top agar thickness. Because of these numerous sources of variation, it is essential that microbial mutagenesis assays be conducted under a Quality Assurance/Quality Control Program with standardized protocols and that each experiment include sufficient positive and negative controls to accurately assess the variability.

The extent of inter- and intralaboratory variation will be an important factor in determining the utility of short-term bioassays as a monitoring tool. The results presented in Table 7 demonstrate the interlaboratory variation with four Salmonella strains and two Bacillus strains for negative controls and diagnostic mutagens for the entire project. For the negative controls, the variability ranged from 14% for TKJ6321 with no additions to 50% for TA1538 with DMSO (Table 7). Cheli et al. (1980) observed that the plasmid containing strains (TA98 and TA100) were generally less variable than the corresponding non-plasmid strains (TA1538 and TA1535), although no such trend was found in this laboratory. While a greater degree of variability was observed with the diagnostic mutagens than with the negative controls, none of the variations were sufficient to influence the final interpretation of the results. That is to say, in all cases, the no-effect dose level and the dose level at which a positive response was obtained were constant. The overall variability of both prokaryotic mutagenesis assays were comparable; however, for the compounds tested, a greater mutagenic response was obtained in strains TA98 and TA100 than in either of the TKJ strains.

The majority of the biological testing performed in this research was conducted with strains TA98 and TA100. The variability of these two strains for each of the three test years is provided in Table 8. Both strain TA98 and TA100 exhibited a broad range of variability, although in general the mean for each year was within one standard deviation of the mean for the other two years. In all three years, the mean spontaneous reversion frequency falls within the range considered acceptable in a survey of eight laboratories conducted by the Environmental Mutagen Information Center (1978).

Periodically during this research, titrations were conducted with diagnostic mutagens in order to evaluate the sensitivity of the tester strains. The results from testing the direct-acting mutagens MMS and MNNG in strain TA100 are provided in Figures 4 and 5. For both compounds on all three test dates, the results are comparable, although a broad range of variation was observed at all dose levels. The results from testing the direct-acting mutagen 2-nitrofluorene with strain TA98

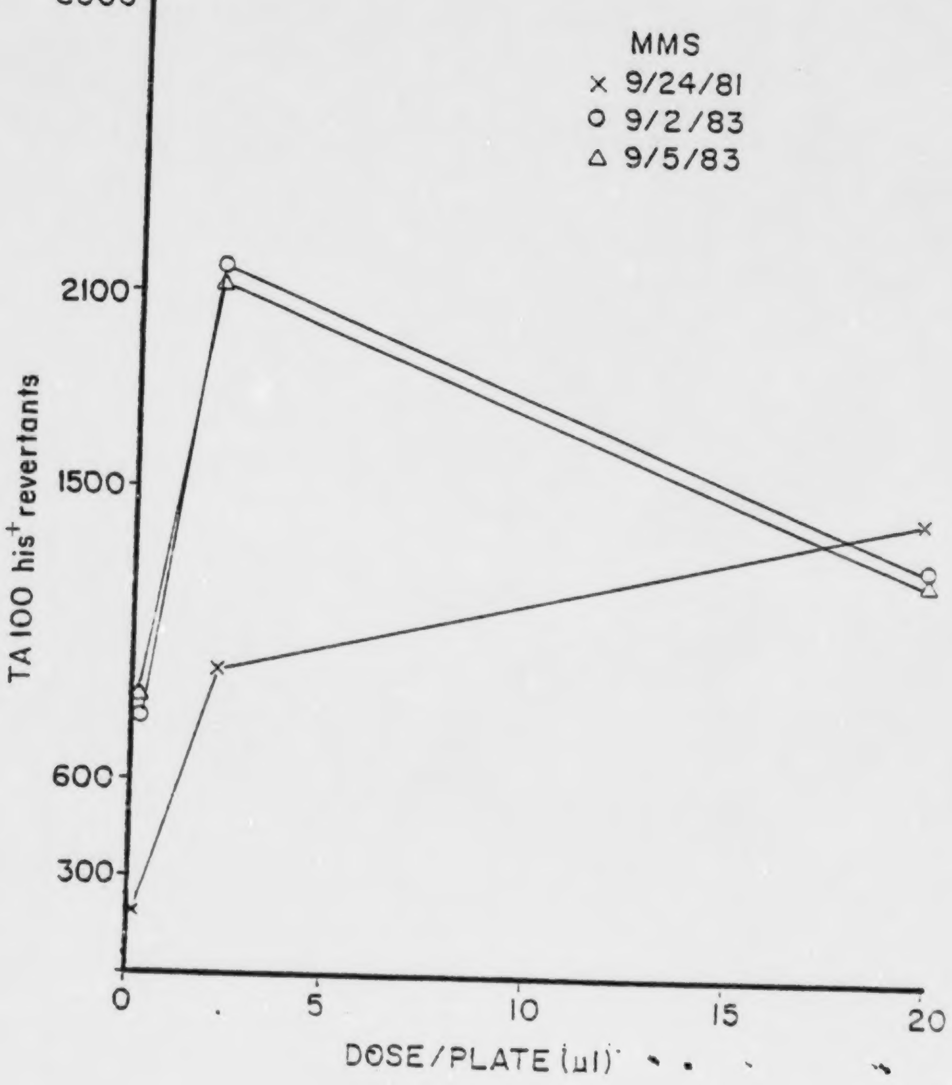

Figure 4. Variability of mutagenic activity of TA100 induced by MMS

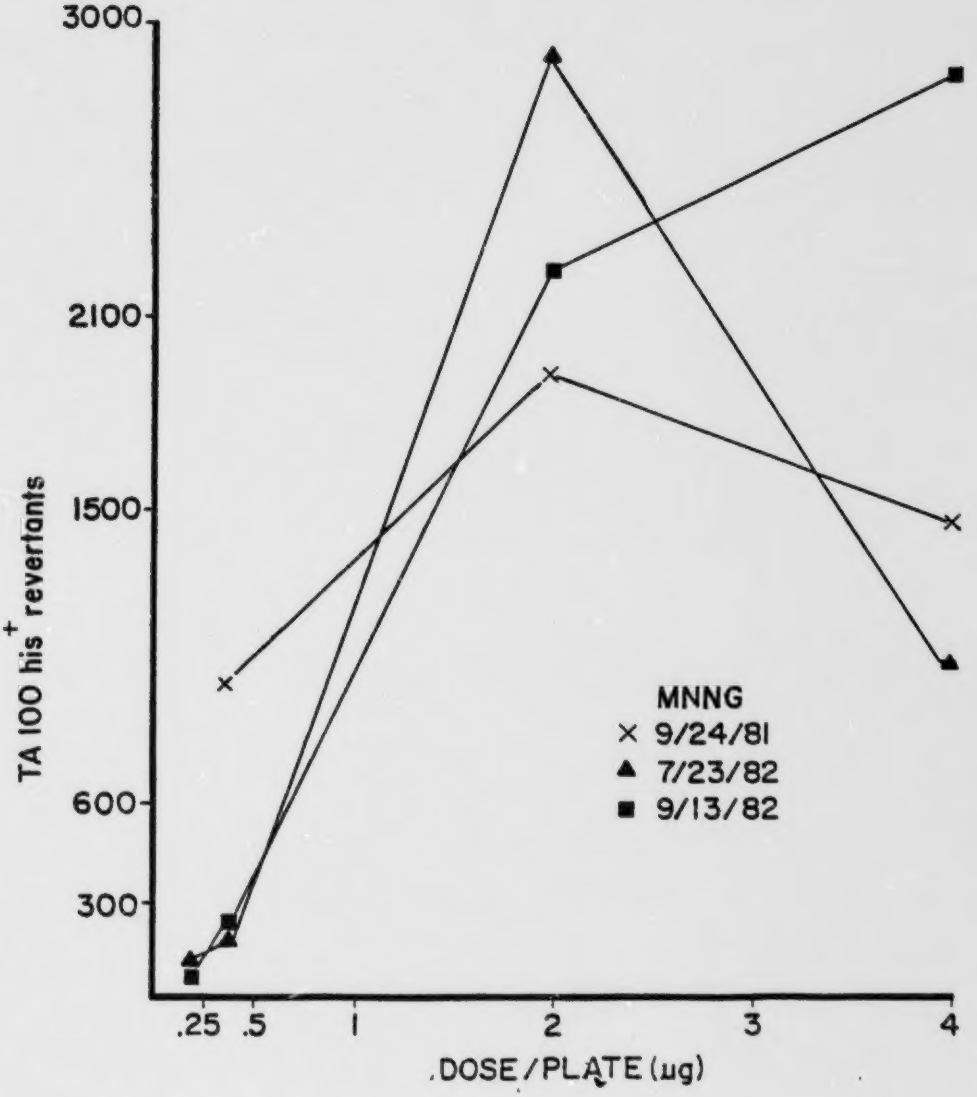

Figure 5. Variability of mutagenic activity of TA100 induced by MNNG.

is provided in Figure 6. While there was a broad range of variability for the 18 month monitoring period, the variability was comparable to that observed by Cheli et al. (1980) over an 11 month period.

The addition of metabolic activation is another parameter which can significantly affect the variability of the Salmonella assay. Cheli et al. (1980) observed that the addition of S9 and 2-aminoanthracene resulted in a broad range of mutagenic responses. The responses obtained with benzo(a)pyrene (B(a)P) and 2-aminoanthracene (2AA) in strain TA100 are given in Figures 7 and 8. The variability of the response from activated B(a)P is smallest at a dose level of 50 µg per plate and increases at higher and lower dose levels. With 2AA, the variability of the responses are greater than for B(a)P, although the optimum dose level was consistently 10 µg per plate. Similar results were obtained with activated B(a)P and 2AA in strain TA98 (Figures 9 and 10). The response induced by B(a)P was less variable than that induced by 2AA; however, the overall mutagenic response induced by 2AA was also greater. For both strains TA98 and TA100, the optimum dose level for B(a)P and 2AA with activation remained constant on all test dates.

An additional comparison was conducted to evaluate the effects of storage on the variability of the mutagenic activity of water extracts over time. The results from two different water extracts tested in three independent experiments at intervals of 2, 5, and 14 months after collection indicate that there was no significant change in the mutagenic activity at all dose levels in either sample (Table 9). There was a large increase in the total induced mutation frequency for both samples with activation on the last test date. However, these results did not deviate significantly from the mean and may have been influenced by the spontaneous reversion frequency that was almost double the spontaneous reversion frequency from the initial test date. Thus, microbial mutagenesis testing has indicated that storage of extracted water samples at 4°C will not appreciably alter their mutagenic potential over a 14 month interval. While retesting did clarify the data interpretation, there was no difference in the final interpretation of the data when compared to the results from each individual testing date. These results, and those discussed previously, emphasize the need for adequate positive and negative controls as well as duplicate experiments in microbial mutagenicity testing. In addition, the Salmonella assay exhibited a wide range of variability over time, although the optimum, toxic, and no-effect dose level remained fairly constant.

The Salmonella/microsome assay is a rapid and simple test procedure that can be used to evaluate the mutagenic and potential carcinogenic activity of test sample. As part of a battery of biological test systems, the Salmonella/microsome assay is considered to be the most widely validated and most sensitive test system (Griem et al., 1980). In addition, this is one of a limited number of bioassays for which a standard protocol has been developed and which has been subjected to numerous intralaboratory evaluations (Dunkel, 1979;

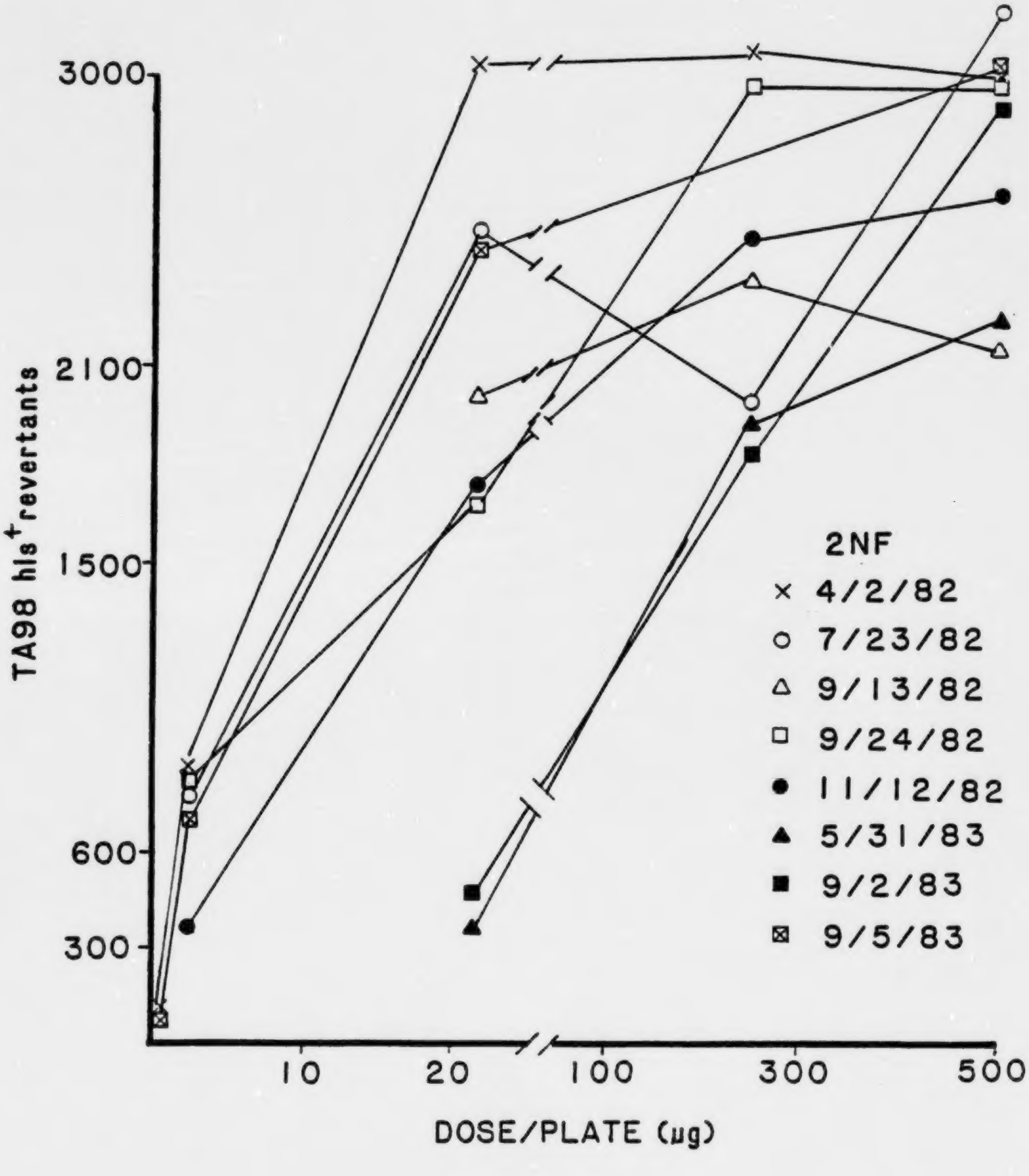

Figure 6. Variability of mutagenic activity of TA98 induced by 2NF.

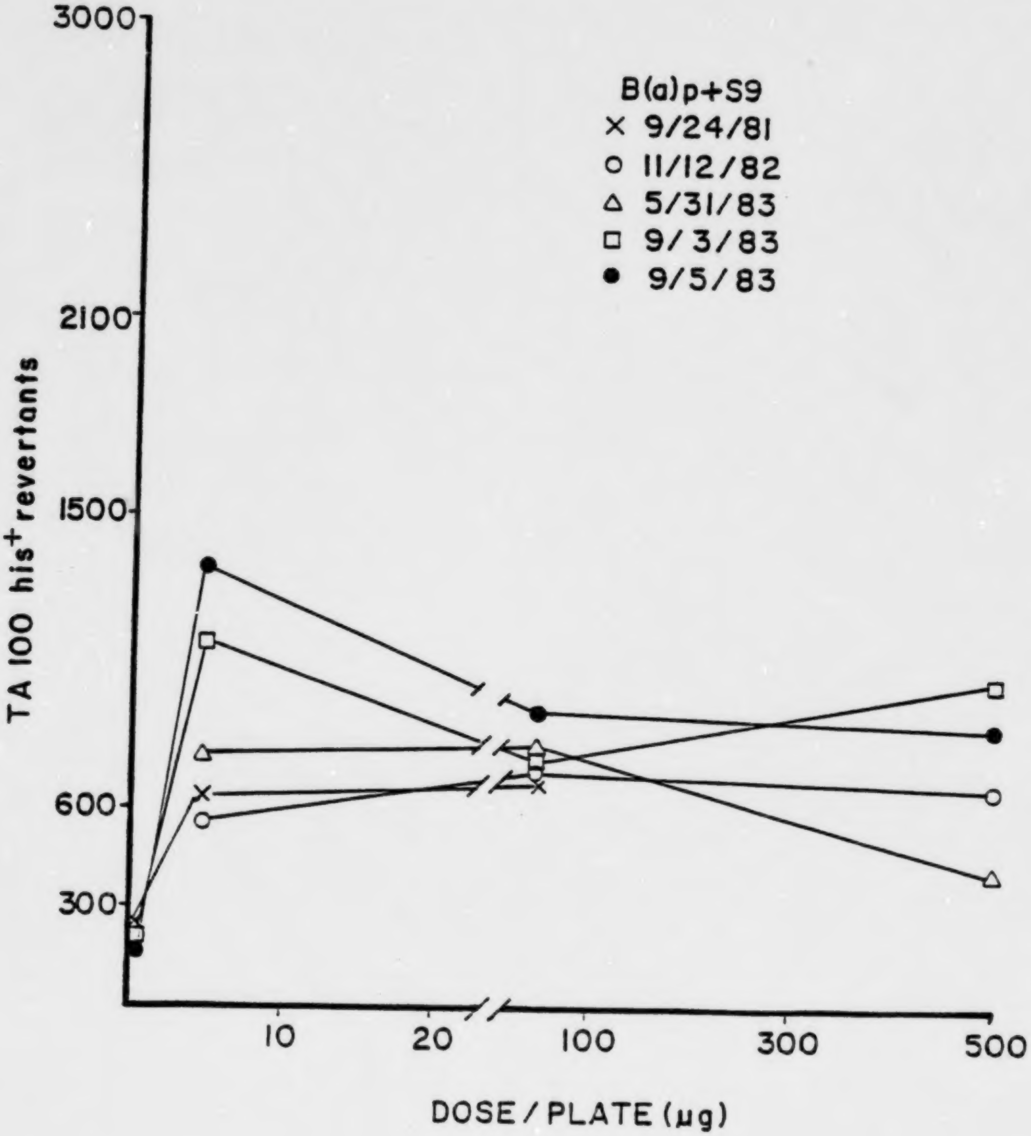

Figure 7. Variability of mutagenic activity of TA100 induced by B(a)P.

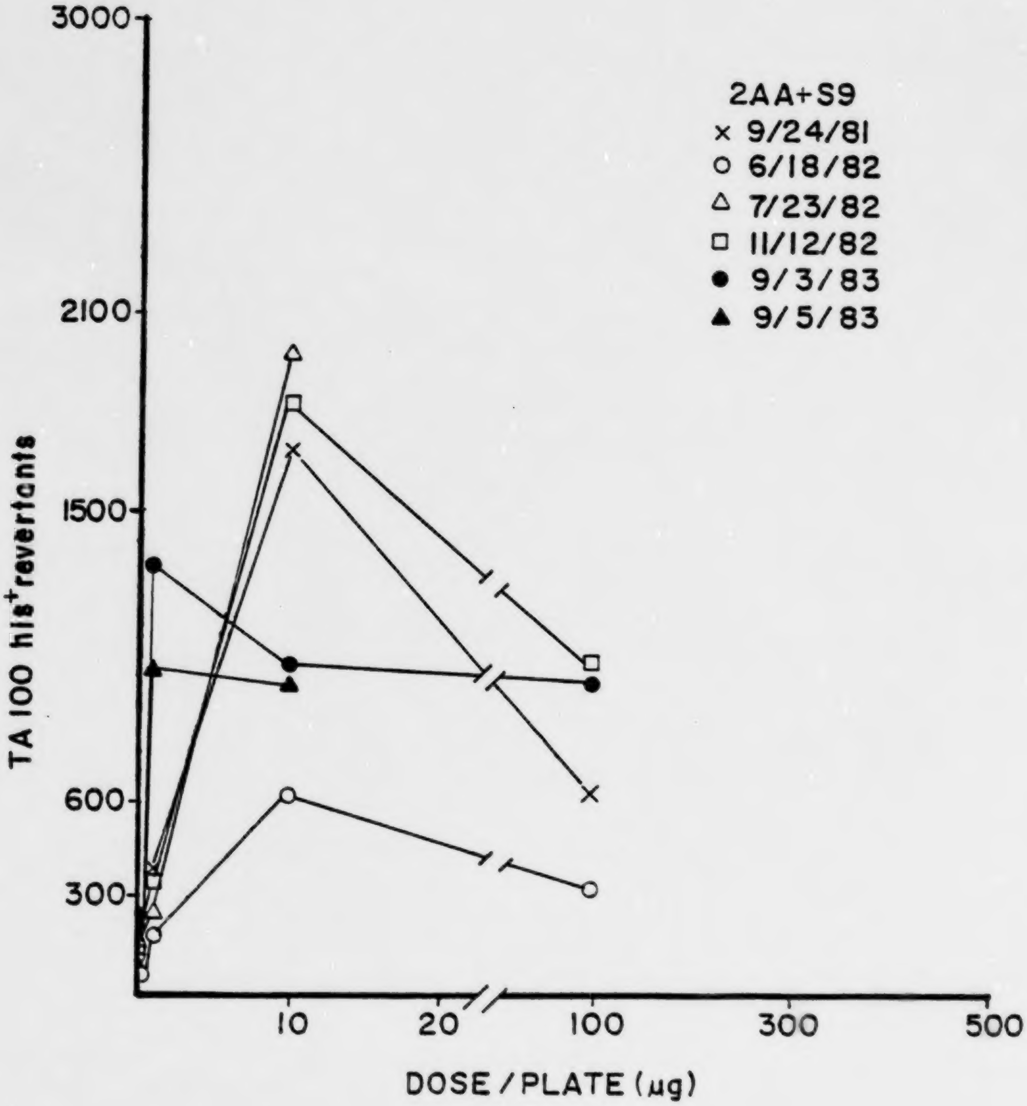

Figure 8. Variability of mutagenic activity of TA100 induced by 2AA.

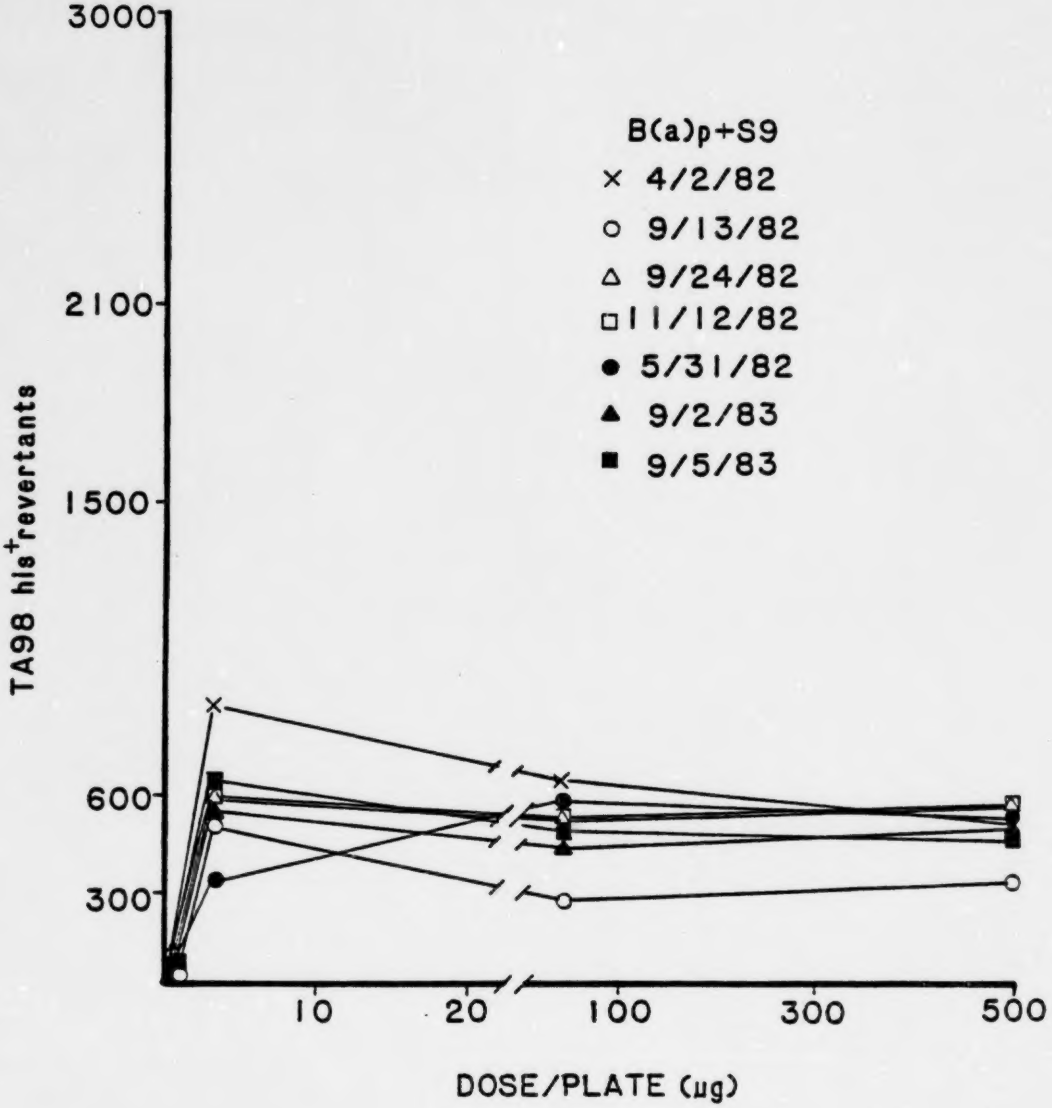

Figure 9. Variability of mutagenic activity of TA98 induced by B(a)P.

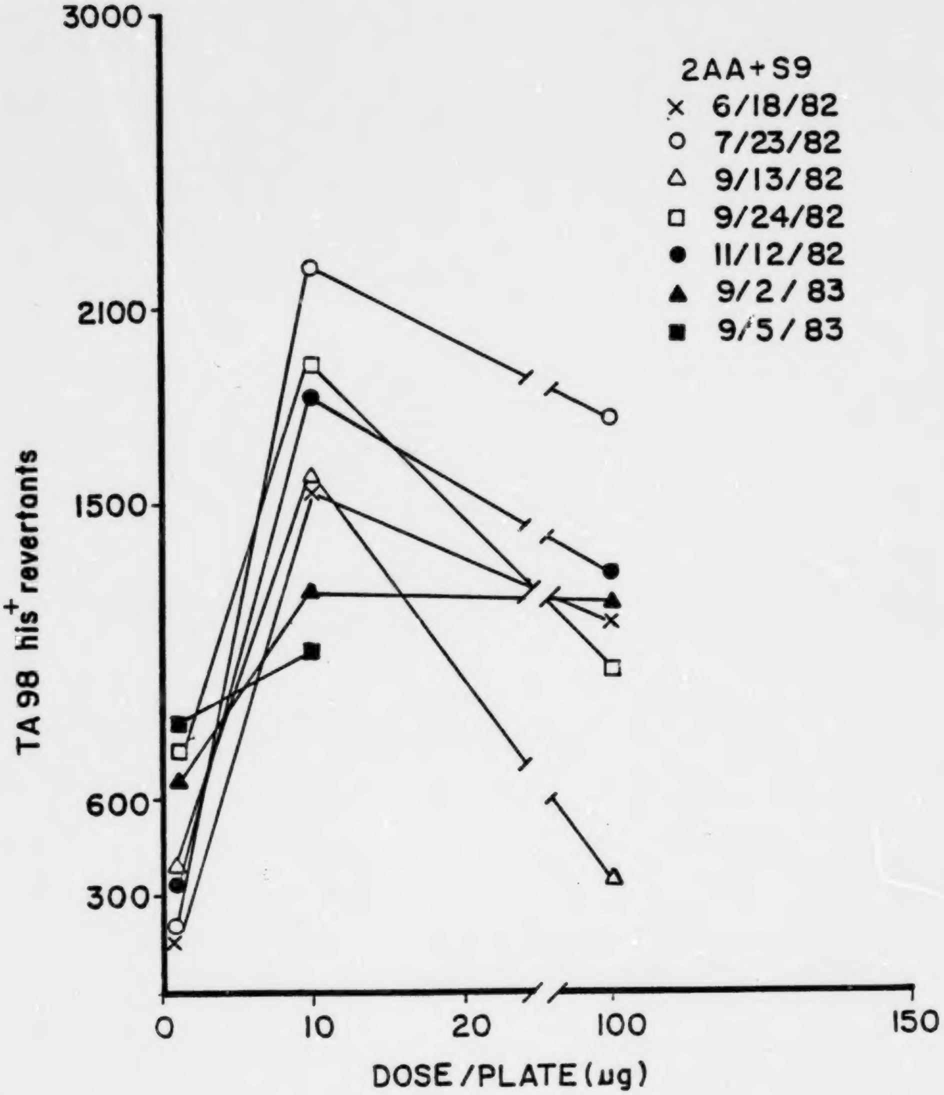

Figure 10. Variability of mutagenic activity of TA98 induced by 2AA.

TABLE 9. THE EFFECT OF STORAGE AT 4°C ON THE MUTAGENIC ACTIVITY OF TWO RUNOFF SAMPLES (SAMPLES WERE RECEIVED 5/13/1982)

Sample	Dose/plate (mg)	TA98 his^+ Revertants*							
		-S9				+ S9			
		7/9/82	10/12/82	7/18/83	Mean	7/9/82	10/12/82	7/18/83	Mean
0582006	0	17	31	34	27	20	39	39	33
	.01	33	18	17	23	54	28	56	46
	.05	34	23	24	27	32	48	58	46
	.1	42	28	42	37	66	65	91	74
	.5	91	70	60	74	129	102	201	144
	1.0	Toxic	Toxic	Toxic	Toxic	120	107	275	167
0582007	0	17	31	34	27	20	39	39	33
	.01	34	18	22	25	22	32	46	33
	.05	28	18	23	23	65	55	56	59
	.1	37	15	24	25	79	73	76	76
	.5	48	28	29	35	145	87	138	123
	1.0	51	26	25	34	115	70	164	116

* Average of two plates.

de Serres and Ashby, 1981; Grafe et al., 1981). Because the plate incorporation assay does not provide data as to the viability of exposed cells, quantitation of this assay is difficult if not impossible (Matney, 1981). As a result, risk assessment from bioassay data is also impossible (Clive, 1980; Griem et al., 1981). Additional limitations of the Salmonella/ microsome assay include its inability to detect volatile compounds, inorganic compounds, and chlorinated hydrocarbons (Brown et al., 1982). However, the Salmonella assay is generally accepted as the primary test in a battery of bioassays because it has a high sensitivity and specificity, and because it is rapid and inexpensive to run (Brookes and de Serres, 1981).

C. Point Mutations and Chromosome Damage: Eukaryotic Organism

1. Test System Description--

Eukaryotic assays for measuring genetic damage are capable of detecting point mutations and small deletions as well as changes in mitotic segregation resulting from induced nuclear damage. Investigations of chemical mutagenesis in haploid Aspergillus nidulans using the methionine assay has been shown to detect base-pair changes and small deletions (Alderson and Hartley, 1969; Hartley, 1969), while investigations using the diploid strains can detect changes in mitotic segregation resulting from crossing-over, non-disjunction, breakdown of mitosis, or chromosome aberrations (Kafer et al., 1982).

The Aspergillus methionine assay detects forward suppressor mutations by measuring colony formation on a media lacking methionine. Restoration of the ability to synthesize methionine in Aspergillus has been shown to arise from a forward mutation in any of six genes (Scott and Alderson, 1971; Lilly, 1965). For the detection of mitotic segregation or chromosome aberrations, diploid conidia heterozygous for recessive color markers, non-utilization nutritional markers, and resistant markers were utilized. Agents inducing mitotic segregation are initially detected by visual observation of phenotypic changes using diploid organisms grown on a complete media that contains the chemicals to be tested. An increased frequency of phenotypic changes, e.g., changes in conidial color or colony morphology, implies that the agent being tested has induced some form of genetic damage.

Although Aspergillus has only been tested with 150 compounds, the same response was obtained in 26 of the 27 compounds for which data from both animal carcinogenesis bioassays and Aspergillus were available. In addition, five of the compounds giving a positive response in the Aspergillus system and carcinogenesis bioassays were negative in the Salmonella assay (Scott et al., 1982). The following is a discussion of the procedures utilized for the haploid and diploid bioassay, and the results obtained with solvent and positive controls.

2. Test System Protocol--

a. Methionine System--The bioassay using the haploid form of *Aspergillus nidulans* has been used to assess the mutagenic potential of various samples by evaluating the induction of mutations at the methionine suppressor loci. The parent strain of *Aspergillus* was obtained from Dr. T. Alderson, MRC Radiobiology Laboratory, Harwell, England. The parent strain was stored in silica gel at 4°C. Stock cultures were obtained by growing *Aspergillus* crystals on complete medium. Working cultures were grown from organisms picked from the stock culture. The requirements for biotin and methionine were checked individually on media lacking the appropriate amino acid, and the untreated experimental controls indicated the presence of an excessive number of methionine suppressor mutants in the conidial suspension before exposure to the test chemical. An acceptable overall spontaneous mutation frequency should be within the range of 2.5 mutants per million conidia assayed. Slightly higher spontaneous mutation frequencies can be expected when conidia are exposed to 37°C or metabolic activation.

Conidia from four to five single colonies of the *meth G1*; *biA1* (requiring methionine and biotin) Glasgow strain of *Aspergillus nidulans* grown for 5 to 6 days on a complete medium (CM) at 37°C were used for each experiment. The conidia were suspended in dilution fluid, i.e., water containing Tween 80, vortexed for 15 min to break up conidial chains, and filtered through sterile cotton to remove mycelial debris. The conidia were then concentrated on a 0.45 μm millipore filter (Millipore-Flouropore filters, Millipore Corp., Detroit, MI), washed with at least 20 ml of dilution fluid, and resuspended in phosphate buffer (pH 7.0) containing Tween 80.

Removal of germination inhibitory substances from the conidia of *Aspergillus* is essential to achieve consistent levels of survival after mutagenic treatments (Scott *et al*., 1972). Inhibitor-depleted conidia were prepared by the addition of a 1% (v/v) solution of diethyl ether to the conidial suspension. The flask containing the 50 ml of conidial suspension with diethyl ether was placed on a shaker for 1 hour at 37°C. The conidia were then collected on a 0.45 μm millipore filter, washed with at least 20 ml of dilution fluid, and resuspended at a density of 240 million conidia/ml in Tween 80 and phosphate buffer (pH 7.0). This method for removal of the germination inhibitor is described in Bertoldi *et al*. (1980).

Test compounds were dissolved in DMSO and added to inhibitor-depleted conidia to yield the appropriate final concentrations of the test chemical. All manipulations and exposures were completed under yellow light. Throughout the exposure, the conidial suspensions were enclosed in a teflon sealed pyrex glass test tube and incubated with mild agitation at a temperature of 37°C for the desired period. The treatment was stopped by the addition of 9 ml of ice cold dilution fluid to 1 ml of the reaction mixture. For the non-activated system, 1 ml of approximately 24 million conidia in Tween 80 phosphate buffer was added to the exposure tube. At the appropriate time, 10 µl of the appropriate concentration of the chemical in DMSO was added to the conidial suspension.

For the activated system, 0.1 ml of approximately 240 million conidia in Tween 80 phosphate buffer was added to the exposure tube containing 0.9 ml of the S9 mix. Thus, the exposure concentration of the conidia was 24 million conidia per ml. At the proper time, 10 µl of the appropriate concentration of the chemical in DMSO was added to the conidial suspension, and the previously described procedure was then followed.

Immediately before and after treatment, the viability and mutability of a conidia population from the biAl, methGl strain of Aspergillus was assayed by platting on the appropriate minimal media. To estimate the viability, minimal medium plates containing methionine, biotin, and sodium deoxycholate were used. Appropriately diluted suspensions were spread over the surface of five plates and incubated for at least two days at 37°C before the viable colonies were manually counted. Media of a similar composition, although lacking methionine and sodium deoxycholate, were used to detect all the methionine suppressors. The methionine-free minimal media was melted in a water bath at 45°C to prevent gelling. Mutants were assayed by pipetting samples of an appropriately diluted conidial suspension into 100 ml of melted minimal media. The resultant suspension was then poured onto 12 petri dishes. The volume added was regulated by the potency of the agent. These plates were incubated for five to six days at 37°C. At this time, the mutants were classified as A (large green colonies), B (brown heavily pigmented colonies), and C (small green colonies with a white hyaline edge) in accordance with the classification system described by Lilly (1965).

For meaningful quantitative data with this system, not only is it important to ensure complete removal of any "germination inhibitory" substance but also to restrict the average total number of methionine mutants per 100 mm diameter petri dish to less than 20 (Scott *et al*., 1973).

For the haploid mutation system, 8-methoxypsoralen (8-MOP) and near ultraviolet light were used for the positive control. In the absence of near UV, 8-MOP can also act as a negative control. The advantage of using this chemical is twofold. First, this particular photosensitizing reaction randomly induces mutation at all loci; therefore, it quantitatively and qualitatively checks the response of the multilocus system, in addition to acting as a positive control. Second, the compound alone exhibits no mutagenic properties with the biAl; methGl strains in the absence of light (Alderson and Scott, 1970; Scott and Alderson, 1971). DMSO exposures under non-growth conditions yield a negative response in this system. This control and the activation mixture control were included in the experimental procedure to ensure that no undetected substances were inadvertently included in the experiment which could lead to a false positive response. Benzo(a)pyrene requires activation in this assay system and was included in the procedure to insure the proper functioning of the activation mixture.

Each piece of raw data was recorded following good laboratory procedures. Raw data was not averaged, transformed, or corrected before recording. Data was recorded in tabular form to indicate numbers of identified mutants and classification of mutants e.g., morphological types. Sufficient detail was recorded so that verification of survival and mutation frequencies could be undertaken if necessary.

The data generated from this study was interpreted in the following four ways. First, data was analyzed by a 3 x N contingency chi square. Second, mutation frequency vs concentration was compared to the spontaneous background mutation frequency. Third, mutation frequency vs exposure time at a fixed sample concentration was compared to background mutation frequency. If the mutation frequency of the latter two analyses exceeded twice the spontaneous level, the test was considered positive. The fourth method of analysis employed an adapted version of the mathematical model of Munson and Goodhead (1977). The points were plotted according to the equation:

$$M-M_o = -m \ln (S/S_o)$$

where:

S_o = population of viable cells at time 0,
M_o = mutation frequency/viable cell at time 0,
S = number of viable cells after exposure to a dose D of the waste fraction in question,
M = mutation frequency/viable cell after exposure to a dose D of the compound in question.

Data were plotted such that the y-axis represents induced mutation frequency (M-Mo) and the x-axis denotes lethal hits ($\ln(S/S_o)$). Linear regression analysis was used to determine the best fit of an unweighted straight line through the data points. If the homogenity as measured by chi square was not significant, the slope of the regression line was obtained. When the slope is less than or equal to 2.5 per million survivors for all suppressor mutants, the compound is regarded as non-mutagenic. If the slope has a value of greater than 5 per million survivors, it is regarded as mutagenic. The values for a negative response of the individual classes of mutants are less than 1.25 per million surviving conidia for A or B and 0.25 for C. Values greater than 2.5 (Class A and B) or 0.5 (Class C) per million surviving conidia are regarded as mutagenic.

b. Diploid Assay--Treatment of heterozygous diploid conidia with mutagens and various chemicals can either increase one of the processes of spontaneous segregation or it can induce mutations that include chromosome aberrations and may lead to apparent increases in segregation. Basically, the four following types of effects can be identified by the relative frequencies and types of stable and unstable segregants: mitotic crossing-over, mitotic non-disjunction, breakdown of mitosis, and mutations with deleterious dominant effects.

The type of mitotic crossing over detected in heterozygous diploids occurs between chromatids at the four strand stage of mitosis. These reciprocal exchanges between markers involving two homologous chromatids can produce complementary diploid recombinants that are homozygous for all markers distal to the exchange, i.e., segregants homozygous for all markers of one chromosome arm. No change in ploidy and viability occurs in this exchange, and the selection is not operative. This type of exchange is usually detected in colonies growing on solid CM by visual identification of segregant sectors that express recessive mutants present in the heterozygous diploid as markers. The most conclusive evidence for mitotic crossing over are twin spots or twin sectors, i.e., recovery of both products of a single reciprocal exchange as paired sectors.

Mitotic non-disjunction of typically single chromosomes produces 2n+1 types in the first instance; in Aspergillus, the reciprocal 2n-1 product is inviable. The trisomic 2n+1 types show no segregation of recessive markers but have a somewhat reduced growth rate compared to the original diploid. Although typically not distinguished from other genetic damage in the original growing conidia, they can be identified through a secondary plating of the original abnormal head. This secondary plating probably increases the rates of secondary non-disjunction or chromosomal loss, as is common for trisomics of many species. The 2n+1 types, therefore, may produce diploid 2nd-order segregants with relatively high frequencies. The random loss of one of the three homologues of a 2n+1 can easily be seen when trisomic conidia are plated at low density. Absence of competition leads to fairly good but not normal growth of the 2n+1 type in the center of colonies. At various points in the colony, faster growing diploid sectors are observed which are due to the loss of the extra chromosome. One third of these diploid improved sectors are showing segregation of a chromosomal type, i.e., all recessive markers of 1 chromosome show up simultaneously in the non-disjunctional diploid sectors. The detailed growth pattern, especially size and conidiation of the trisomic center, are different and characteristic for each of the 8 2n+1, trisomics for any one of the 8 linkage groups or chromosomes. Each can be identified on standard media if no chromosomal aberrations are present. For spontaneous mutation frequencies, non-disjunction is the main process producing spontaneous haploids, which are rarer than non-disjunctional 2n segregants; these, in turn, are 5 to 10 times less frequent than 2n mitotic crossing over. For induced mutation frequencies, these ratios are altered.

Misdistribution of chromosomes leading to haploidization rarely occurs but is a frequent effect of treatments with spindle poisons. If the products of single treated nuclei are analyzed carefully, a large number of somewhat-related segregants are found, all of them showing chromosomal type segregation. These include any aneuploid types that are viable, i.e., 2n+1 up to 3 and n+1 up to 3. Specific aneuploid types are recognizable only if conidia from the most abnormal-looking areas are replated at low density. The stable types, however, are mainly haploids, in addition to some "non-disjunctional" diploids, i.e., diploid segregants homozygous for all markers of 1 or several chromosomes. Haploids are the most viable and best growing types if mitotic inhibitor is present during growth of the mycelium. Such agents are, therefore, used for haploidization of diploids in order to verify genotypes or detect translocations and recessive lethals.

Induced mutation with dominant effects on viability, semi-dominant lethals, deletions, and other unbalanced aberrations, lead to formation of abnormal colonies. The original unbalanced nuclei are at a disadvantage, resulting in poor growth and the formation of few conidia even if plated at low density. However, as is the case of aneuploids, the original unbalanced nuclei spontaneously produce well-growing 2nd-order segregants, either by mitotic crossing over, non-disjunction, chromosome loss, or any combination of these. In competition with the growing mycelia of normal nuclei, only the euploid final segregants will be recovered from the original unbalanced nuclei. These induced mutations are of the same type as produced by the three preceeding mechanisms and vary primarily in relative frequencies. Increase of haploid and diploid crossing over is usually diagnostic for chromosomal aberrations, since none of the preceeding three types of effects produce both types of segregants simultaneously.

Mutations in diploids that result in recessive lethals or balanced translocations will predominantly produce no obvious change in phenotype. In other words, there will be no change in morphology or growth, nor any detectable segregation of markers. Such mutations can, however, influence the recovery of haploid types when these are selected from heterozygous diploids. Any recessive lethal on a specific homologue prevents recovery of the marker located on this chromosome. Balanced translocations generally cause complete linkage of markers that are located in the two involved chromosomes.

Diploid strains of Aspergillus are not completely stable; test diploids must be synthesized fresh from heterokaryons between 2 suitable haploid components. This has the advantage that strains are easily tested for all markers, except for certain cases of epistatic types, using standard procedures.

c. Diploid strains for analysis of induced segregation--There are two main considerations in the construction or choice of test diploid that work in the opposite directions. First, a minimum of testing should be needed for the identification of small increases in the frequencies of the three main types of stable segregants so that large numbers can be handled for preliminary screening. Secondly, a diploid should contain enough markers for detailed analysis of the type of induced genetic damage. Ideally, the diploid should have the following features:

a. Markers to distinguish all haploids from diploids by simple tests;

b. Markers on both arms of one or more chromosomes to distinguish diploid crossing over from diploid "non-disjunctional" types;

c. Two recessive color markers on the same chromosome arm, heterozygous in repulsion, to identify twin sectors; these may also permit visual identification of certain haploids from diploid segregants;

d. One marker in each linkage group to check segregation of the 8 different chromosomes in haploids;

e. Markers on each homologue, or semi-dominant markers in each group to follow all chromosomal segregation in diploid, as well as haploid segregants;

f. Many markers on both arms of at least one linkage group to follow segregation patterns after induction of chromosome aberrations or semi-dominant lethals.

New diploids are constantly being constructed to meet the above requirements. The diploid used in this investigation was Diploid 20, a cross of haploids F475 x F513 (Figure 11). Haploid strains were obtained from Fungal Genetic Stock Center (FGSC, Arcadia, California).

Data from the diploid assay is expressed as the induced segregation index with respect to the negative control or the solvent control. The induced segregation index is calculated by dividing the frequency of mutations induced by a specific sample by the spontaneous mutation frequency (negative control). A response is considered positive if the induced segregation index is statistically greater than 1.0. Following treatment of heterozygous diploid conidia, the abnormal or segregating colonies are replated to obtain secondary segregation or pure primary segregation, respectively. These isolates are further characterized by replicate plating onto various nutritional and resistant media.

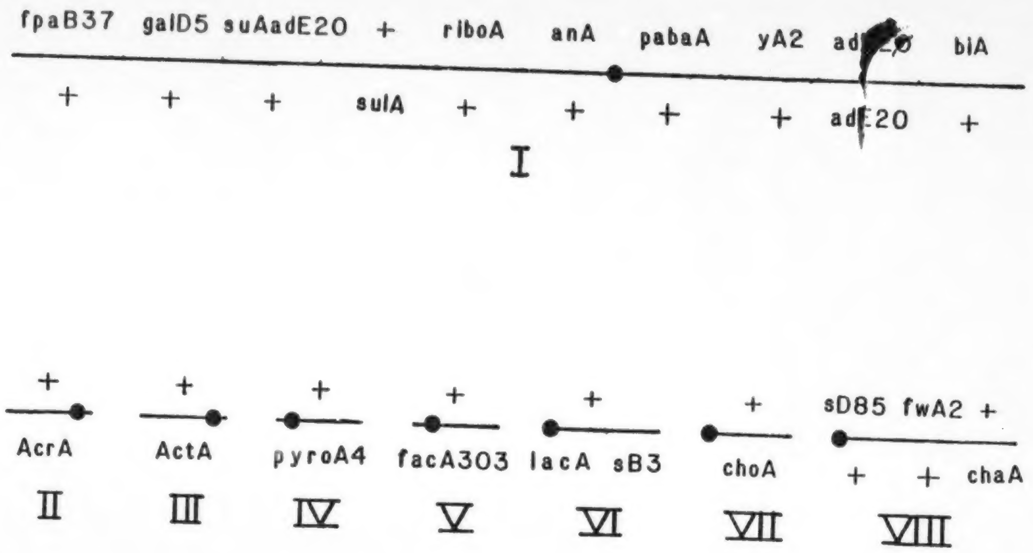
fpaB37
galD5
suAadE20
+
riboA
anA
pabaA
yA2
ad[illegible]
biA
+
+
+
sulA
+
+
+
+
adE20
+
I
+
AcrA
II
+
ActA
III
+
pyroA4
IV
+
facA303
V
+
lacA
sB3
VI
+
choA
VII
sD85
fwA2
+
+
+
chaA
VIII

Figure 11. Genotype of *Aspergllus nidulans* diploid 20.

The nature of the genetic damage can then be determined by the response on the various nutritional and resistant media. The mutation frequency for each class and overall genetic damage is then determined by dividing the number of colonies in each class by the total number of colonies examined for each exposure.

3. Test System Results (Eukaryote)--

Two groups of compounds were used as solvent and positive controls in the haploid and diploid assays using *A. nidulans*. In the haploid methionine system, DMSO and 8-MOP served as negative controls, where as 8-MOP+NUV and benzo(a)pyrene (B(a)P) were positive controls. Following exposure to 10 µl of the solvent DMSO for 60 min, the frequency of induced mutations per million surviving conidia for colony types A, B, C, and the total were 0.47, 0.21, 0.041, and 0.72, respectively (Table 10). For the 8-MOP in the absence of near ultraviolet (NUV) light, the total induced mutation frequency was $1.4/10^6$, and the induced mutation frequencies for class A,B, and C were 0.69, 0.48, and $0.24/10^6$, respectively (Table 10). Mutations were randomly induced at all loci when the 8-MOP was photoactivated with near UV light. The total induced mutation frequency was 250 per million surviving conidia. In addition, activated 8-MOP induced 89, 88, and 70 mutations/10^6 surviving conidia in classes A,B, and C, respectively (Table 10). Benzo(a)pyrene in the presence of a rat liver activation system induced 16 type A and B, 8 type C, and 40 total mutations per million survivors (Table 10). The standard deviation of the induced mutation frequencies for these controls observed over the three years of testing of DMSO, 8-MOP, 8-MOP+NUV, and B(a)P were 94, 69, 18, and 50%, respectively. In most cases, the greatest deviation was observed in class C colonies. In no case, however, was the deviation sufficient to alter the interpretation of the results.

The negative control used in the diploid assay was the top agar alone, while DMSO served as a solvent control, and benomyl was the positive control. The results from the negative and positive controls for the segregant and abnormal colonies recovered from the diploid *A. nidulans* are summarized in Tables 11 and 12. The induced segregation index for the segregant colonies with respect to DMSO was 0.04 for the negative control and 3.7 for benomyl. While the induced segregation index for DMSO and benomyl with respect to the negative control was 1.4 and 5.2, respectively. Benomyl induced genetic damage at all loci in both segregant and abnormal colonies. The overall mutation frequency observed in the abnormal colonies induced by benomyl was .157, which is equivalent to an induced segregation index that is approximately four times greater than both the solvent and negative controls. Significant increases were observed in all classes of abnormal colonies induced by benomyl, except the hyperhaploid class.

The two bioassays using *Aspergillus nidulans* as an indicator organism to detect primary genetic damage provide information not

TABLE 10. SURVIVING FRACTION AND INDUCED MUTANTS PER SURVIVOR IN HAPLOID A. NIDULANS FOLLOWING TREATMENT WITH SOLVENT AND POSITIVE CONTROLS

Chemical	Dose/pl (µg)	Exposure Time (min)	Number of Observations	Surviving Fraction (Mean ± SD)	Colony Type	Induced Mutations/ Survivor X 10^{-6} (Mean ± SD)
DMSO	10 µl	60	14	.98 ± .02	A	.47 ± .33
					B	.21 ± .37
					C	.041 ± .057
					T	.72 ± .68
8-MOP	50	90	14	.99 ± .05	A	.69 ± .59
					B	.48 ± .46
					C	.24 ± .24
					T	1.40 ± .97
8-MOP+NUV	50	5	14	.47 ± .13	A	87.0 ± 14.0
					B	88.0 ± 17.0
					C	70.0 ± 16.0
					T	245.0 ± 45.0
B(a)p	11.6	20	14	.52 ± .03	A	16.0 ± 6.9
					B	16.0 ± 8.5
					C	8.0 ± 4.7
					T	40.0 ± 20.0

TABLE 11. SUMMARY OF GENOTOXIC EFFECTS INDUCED BY CONTROLS IN SEGREGANT COLONIES OF DIPLOID A. NIDULANS

Sample	Total[1]	Overall	Phenotype[2,3]											ISI[4]	
			Yellow			Fawn		CHAT		DG		GR			
			CO	ND	M	2n	n	2n	n	2n	n	2n	n	CT	DO
Control	619	1.9	0.3	0.5	0	0.3	0	0.3	0	0.3	0	0.2	0	–	.04
DMSO	140	4.0	0.7	0.7	0	0	0.7	2.0	0	0	0	0	0	1.4	–
Benomyl	299	12.7	1	3.5	0.3	1.4	0.3	2.3	0.7	1.0	1.3	1.0	0	5.2	3.7

1. Total = number of segregant colonies examined; overall = total number of induced segregants.
2. Units = total frequency of segregants per colonies examined.
3. Phenotype: Yellow-includes only colonies that were completely yellow, all other listed in other sections; CO = crossing-over, ND =non-disjunction, M = mutations, Fawn = tan sectors; CHAT = chatreuse; DG = dark green G = green; 2n = diploid; n = haploid.
4. Induced segregation index = Total induced segregants divided by frequency of control segregants. CT = evaluated with respect to controls; DO = evaluated with respect to solvent control (DMSO). If ISI is significantly greater than 1.0, response is considered positive.

TABLE 12. SUMMARY OF GENOTOXIC EFFECTS OBSERVED IN ABNORMAL COLONIES OF DIPLOID A. NIDULANS FOLLOWING EXPOSURE TO CONTROLS

		Genetic Damage Observed[1]					
Sample	Total[2]	OV	HD	NH	HH	BK	OT
CONTROL	COL=619	22	14	3	2	1	2
	MF	.036	.023	.005	.003	.002	.003
	ISI(S)	-	-	-	-	-	-
	ISI(D)	-	-	-	-	-	-
DMSO	COL=140	6	3	2	0	0	1
	MF	.043	.021	.014	-	-	.007
	ISI(S)	1.19	.91	2.8	-	-	2.3
	ISI(D)	-	-	-	-	-	-
BENOMYL	COL=299	47	19	3	1	19	5
	MF	.157	.050	.010	.003	.064	.017
	ISI(S)	4.36	2.17	2.0	1.0	32	5.67
	ISI(D)	3.65	2.38	.71	-	-	2.43

1. Genetic damage observed: OV = overall summary of genetic damage observed in all categories; HD = hyperdiploid, results from non-disjunction of chromosomes; NH = near hyperdiploid, probably breakdown products including a mixture of haploid and diploid colonies; HH = hyperhaploid, results from major deletions or lethals in chromosomes; BK = breakdown, results from mitotic spindle poisons; OT = others, results from mitotic recombination which has occurred prior to aneuploidy.

2. Total: col = total number of colonies examined, and total number of abnormals in each class; MF = total mutation frequency = OV COL; ISI(S) = induced segregation index with respect to control = MF (sample) MF (control); ISI(D) = induced segregation index with respect to solvent control = MF(sample) MF(DMSO).

available from the bioassays using B. subtilis or S. typhimurium. The methionine system employs a eukaryotic organism to detect forward mutations, and the diploid assay provides additional information on chromosome damaging agents. While the variability of the negative and solvent control was quite high, the spontaneous mutation frequency is extremely low and the frequency of mutations induced by the positive controls is extremely high. The low background mutation frequency observed in Aspergillus bioassays does not overwhelm the response as would occur in a test system with a high background. A compound inducing a weak response might go undetected in a test system with a higher background frequency. Aspergillus, because it can be used in a haploid or diploid form, can detect a broad range of genetic damage and, as a result, detects a range of genotoxic compounds.

The primary limitation of the Aspergillus bioassays is the complexity of the test protocols. The time and expenses incurred in conducting a complete Aspergillus testing protocol will restrict its utility as part of a routine monitoring program. However, Aspergillus is a critically important segment of the initial waste screening protocol because it is one of the least complex and most widely validated test systems used to detect chromosome damage.

D. Evaluation of the Battery of Test Systems

Four different biological test systems have been evaluated in this research to determine their utility to monitor the environmental impact of land treatment of hazardous industrial waste. The DNA repair spot test is severely limited by problems with compounds that are insoluble in water or require metabolic activation to reach their ultimate mutagenic form. The modified plate incorporation assay (Donnelly et al., 1983) enhances the sensitivity of the DNA repair assay but also increases the cost per sample. However, since DNA repair assays are capable of detecting mutagenic compounds not detected by the Salmonella assay (Shiau et al., 1980), they do provide a useful compliment as part of a battery of bioassays. Thus, the DNA repair plate incorporation assay would be useful in a waste characterization protocol, while the utility of the spot test would be limited to a monitoring program provided its sensitivity to the anticipated mixtures has been previously demonstrated.

The Salmonella/microsome assay is perhaps the least complex and most widely validated of all the microbial mutagenesis test systems. This bioassay is known to have a high degree of sensitivity and specificity (Bridges et al., 1981), while it is insensitive to a number of compounds that might be anticipated in a hazardous waste (Rinkus and Legator, 1978). Although a negative result in a bacterial mutagenesis assay is not a definitive indicator of non-carcinogenicity due to the existence of non-mutagenic carcinogens, this test can serve as an extremely effective screening test for those compounds which are carcinogenic by causing damage to DNA (Bridges et al., 1981). The bacterial mutagenesis assay using B. subtilis did not appear to enhance the sensitivity of the battery of bioassays, although it was only used to test a limited number of samples. The Bacillus mutagenesis assay has been found to be sensitive to compounds not detected in the Salmonella assay (Shiau et al., 1980) and may be applicable to certain types of waste not evaluated in the present study. This research and an earlier evaluation of a battery of bioassays by Ashby (1981) confirmed that the Salmonella/microsome assay is best-suited as the primary test system.

Ashby (1981) also recommended that the bioassay used to compliment the Salmonella test be based on a eukaryotic system. The two eukaryotic bioassays utilizing A. nidulans appear to offer the maximum specificity and sensitivity of all the test systems evaluated in the present research (Scott et al., 1982). Brookes and de Serres (1981) suggest that there are two critical requirements for a test to compliment the Salmonella assay. These include a high specificity in order to prevent increasing the incidence of false positives and the ability to detect those carcinogens missed by Salmonella. As a compliment to the Salmonella assay, the Aspergillus bioassay meets both these requirements. The primary utility of the Aspergillus bioassay lies in its ability to detect compounds and types of genetic damage not detected in the prokaryotic systems. Because of the complexity of the Aspergillus test protocols, the utility of this system is limited if a mutagenic sample is also detected in a prokaryotic assay.

The battery of bioassays that is employed to monitor hazardous waste disposal should be selected through the evaluation of several factors. The test protocol used to characterize a hazardous waste should include test systems capable of detecting DNA damage, point mutations, and chromosome damage. The composition of the battery of biological test systems used to characterize a waste should be determined according to the number, structural type, and anticipated environmental fate of the hazardous waste constituents. Brookes and de Serres (1981) suggest that by considering these factors when selecting a battery of bioassays, sensitivity (mutagenic carcinogens/total carcinogens) can be as high as 100%, while specificity (nonmutagenic noncarcinogens/total noncarcinogens) would be no less than 95%. As the waste characteristics are defined and the fate of waste metabolites is better understood, the intensity of the testing protocol can be reduced. Ultimately, once the lab and field evaluations have been completed, the monitoring of a hazardous waste land treatment facility should be feasible using only one of the more simple spot tests.

SECTION 5

WASTE CHARACTERIZATION

INTRODUCTION

Industrial wastes, defined by regulations as hazardous, may contain chemicals that are ignitable, reactive, corrosive, or toxic (CFR, 1980). The present study has employed short-term bioassays to detect mutagens, as well as other potentially genotoxic agents present in waste fractions. Genotoxic compounds constitute the relatively small group of agents that are highly specific for nucleic acids and produce deleterious effects in genetic elements at subtoxic concentrations (Brusick, 1980).

All current waste disposal techniques, e.g., incineration, landfilling, deep well injection, and land treatment, could potentially result in the release of genotoxic compounds into the environment. Estimates as to the environmental origin of cancer range from 60 to 90% (Epstein, 1974). While much of the environmental cancer may result from individual exposures, e.g., smoking, drugs, alcohol, etc., the disposal of hazardous waste should be carefully monitored, in order to minimize any additions to this burden.

The first step in monitoring any method of waste disposal is to accurately assess the hazardous characteristics of the waste. The analytical methodology that is currently being utilized to characterize hazardous wastes employs only a chemical analysis. Chemical analysis alone is insufficient to evaluate the hazardous characteristics of a waste. Component identification of a complex mixture cannot account for potential interactions that may occur between components of the mixture or the waste and soil components. Interactions between waste and/or soil components may be synergistic, additive, or antagonistic. Biological analysis can be used to evaluate the toxic potential of the waste as a whole and the subfractions of a waste or a waste-soil mixture. Chemical analysis can be used to supplement the results of biological testing in order to define the nature of toxic constituents and verify the absence of artifacts generated in the collection or extraction process. Bioassay directed chemical analysis provides a management tool which can be used to make semi-quantitative risk assessments when the results are compared to reference compounds, such as cigarette smoke condensate (Barnes and Klekowski, 1978).

This research has been conducted to evaluate the mutagenic potential of three hazardous industrial wastes. Microbial bioassays capable of detecting point mutations, lethal damage to DNA, and chromosome damage have been conducted on the subfractions of the three wastes. Since the composition of DNA in microorganisms is the same as that in humans, these test systems are anticipated to provide an indication of the potential of waste constituents to cause genetic damage in the human population.

MATERIALS AND METHODS

Wastes

A group of thirteen wastes were initially collected for use in the project. These included two wood-preserving wastes, four refinery wastes, four petrochemical wastes, a pulp and paper waste, an alum sludge, and a paint sludge. A listing of these wastes is given in Table 13. Ten wastes were selected for acute toxicity analysis, and three of the ten were selected for use in the waste characterization and greenhouse studies. Waste selection for the characterization and greenhouse studies was based on the results of a chemical characterization and the results from the acute toxicity study. Each waste was collected on site by study personnel. A permit was obtained from the Texas Department of Water Resources to allow for the collection, transport, and storage of hazardous waste.

Prior to departure for collection of the waste, materials were assembled for waste collection, personnel protection, and waste storage. Although most wastes were collected in-line at a refinery or petrochemical plant, shovels, buckets, and a sludge pump were transported to each site in order to facilitate waste collection. Protective devices included respirators, gloves, goggles, and disposable clothing. The collected waste was placed in a 55 gallon barrel. Each drum was washed with soap and rinsed with water before use. Two barrels containing approximately 50 gal of each waste were obtained. Upon returning to the laboratory, a 4 l reserve sample of each waste was collected and stored at $0^{\circ}C$.

The wood-preserving bottom sediment, PENT S, waste was collected from a wood-preserving plant utilizing both pentachlorophenol and creosote as preservative agents. The effluent from the treatment process was pumped from a concrete storage basin to a large lagoon where the solids were allowed to settle-out while the liquids evaporated. At the time the waste was collected, the lagoon had been in use for several years, and the bottom sediment was approximately .9 meters in depth. The ultimate planned disposal technique for this waste was to dredge the lagoon and remove the sediment to a landfill. There are, however, six existing facilities in the U.S. utilizing land treatment for disposal of wood-preserving waste (Brown and Assoc., 1981). Hazardous constituents anticipated in the PENT S waste include phenols, polycyclic aromatic

TABLE 13. GROSS CHARACTERISTICS OF HAZARDOUS WASTES COLLECTED FOR STUDY

Waste	EPA No.	Extractable[1] Hydrocarbons (%)	Physical[2] Form	Use in Study[3]
Wood-Preserving Bottom Sediment (PENT S)	K001	27	Sludge	A,W,G,L
Wood-Preserving Wastewater	-	NT	Liquid	A
Slop-Oil Emulsion Solids	K049	86	Liquid	A,W
Combined API Separator, Waste Treatment Sludge (COMBO)	K051	41	Sludge	A,W,G
Storm-Water Runoff Impoundment (SWRI)	-	21	Sludge	A,W,G
Dissolved Air Flotation	K048	5	Sludge	A
Acetonitrile	K013	2	Liquid	A,W
Methyl Ethyl Ketone	-	97	Liquid	A,W
Phenol production	-	0.2	Liquid	A
Agricultural Chemicals - Biosolids Waste	-	0.2	Liquid	A
Primary Clarifier Pulp and Papermill		NT	Solid	N
Alum Sludge		NT	Liquid	N
Paint Sludge		NT	Sludge	N

1- Percent by weight, extracted with dichloromethane; NT = not tested.
2- Physical form estimated from visual observation.
3- N=Not used; A=acute toxicity; W=waste characterization; G=greenhouse; L=lysimeter.

hydrocarbons, pentachlorophenol, other chlorinated hydrocarbons, and dioxins. Although the volume of wood preserving sludge produced by the entire industry each year is less than 2,000 dry metric tons, these sludges contain compounds that are toxic even at extremely low dose levels. This waste was utilized in the acute toxicity, waste characterization, greenhouse, and lysimeter studies.

A second wood-preserving waste was collected from a facility using both creosote and pentachlorophenol as preservative agents. This waste was collected on site by pumping the effluent from a lagoon used for spray irrigation. Being a wastewater and not a sludge, this waste was only used in the acute toxicity study.

The slop-oil emulsion solids waste was collected from an API oil-water separator at a petroleum refinery. The waste is generated from skimming the emulsified oil-water layer from an API separator; it was collected by pumping the waste from the pit into a tank truck and discharging from the tank truck into collection barrels. More than 31,000 metric tons of hydrocarbons are generated in the U.S. from this waste stream each year (Abrams et al., 1976). Slop-oil emulsion solids typically contain 40% water, 43% oil, and 12% solids. This waste is listed as hazardous because significant quantities of lead and chromium may be encountered in the waste. The sample collected for the current study was a liquid waste with a low solids content and composed of 86% extractable hydrocarbons. Considering the separation process from which this waste was generated, the slop-oil emulsion solids should be composed of predominantly low molecular weight hydrocarbons. A waste that is composed of predominantly saturated or other low molecular weight hydrocarbons should degrade quite rapidly following land application. The slop-oil emulsion solids waste was only used in the acute toxicity study.

The second refinery waste consisted of the combined waste streams from the API-separator sludge and slop-oil emulsion solids. The sludge was being stored in a two acre lagoon. The waste was collected from the lagoon with a backhoe and placed in the barrels for transport. The API separator sludge and slop-oil emulsion solids account for 13.9 and 18.6% of the total hydrocarbons in refinery solid waste, respectively (Abrams et al., 1976). Waste streams of this nature are currently being treated by land application at a large number of facilities in the U.S. (Brown and Assoc., 1981). Anticipated mutagenic constituents in this waste include polynuclear aromatic hydrocarbons and heterocyclic nitrogen containing compounds. The combined API-separator and slop-oil emulsion solids (COMBO) waste was used in the acute toxicity, waste characterization, and greenhouse studies.

The third waste common to petroleum refineries that was collected and used in the study was a storm-water runoff impoundment (SWRI) waste. This waste is generated from plant housekeeping and rainfall runoff from the plant site. All runoff water from the refinery from which the sample was collected was channeled into a large basin. Two 189.3 l samples were collected on-site from the settling basin from which the sludge was periodically removed for land disposal. The sludge was collected at a location on the side of the settling basin where the water had evaporated and was shoveled into the collection barrels. Storm-water runoff silts account for more than 12,000 metric tons of hydrocarbons in refinery solid wastes each year (Brown and Assoc., 1980). The collected sample contained 21% extractable hydrocarbons. As this waste was generated from runoff and wash water, the composition of hydrocarbons in the SWRI waste was be more varied than the other refinery wastes. In addition, because this waste was contaminated with soil particles from the refinery, it may have had an indigenous microbial population that could enhance waste degradation. The SWRI waste was used in the acute toxicity, waste characterization, and greenhouse studies.

The fourth refinery waste collected for the present study was a dissolved air flotation float (DAF) waste. The DAF waste was obtained from a truck load of waste at a land treatment facility. This waste contained the finely divided oil, clay, and silt particles that were not settled out in the API-separator. The DAF waste is listed as hazardous because of its lead and chromium content. A typical DAF waste consists of 82% water, 12.5% oil, and 5.5% solids. The collected sample contains 5% extractable hydrocarbons. The DAF waste was only used in the acute toxicity study. Each of the four refinery sludges collected for the present study are amenable to land treatment because of their relatively low concentration of toxic constituents. These wastes are produced in large volumes and are currently being treated by land application at existing facilities. Thus, two of the refinery wastes, SWRI and COMBO, have been used in the waste characterization and greenhouse studies.

Four wastes were collected from petrochemical plants. The bottom stream from the acetonitrile purification column (ACN) is a very low solids, low organic liquid waste from the production of organic chemicals. This waste was collected in-line from the acetonitrile purification column using equipment supplied by plant personnel. An on-site GC analysis of the collected sample indicated a composition of 3.5% acetonitrile, 0.1% phenols, 0.9% acetamide, 0.5% heavy ends, and approximately 95% water. The extractable hydrocarbon content of this waste was found to be 2%. Anticipated toxic constituents include hydrocyanic acid and polynuclear aromatic hydrocarbons (Lowenback *et al*., 1978). Because the physical state of this waste would limit land treatment, the ACN waste was used exclusively for the acute toxicity study and characterized using the *Salmonella*/microsome assay.

The second waste from the production of organic chemicals was the methyl ethyl ketone (MEK) waste. The U.S. production of methyl ethyl ketone in 1978 was 300 million kilograms (Beck, 1979). This waste is a very viscous liquid containing 97% extractable hydrocarbons. Since the MEK waste may contain as much as 40% sulfuric acid, land application must be precluded by a pH adjustment. The amenability of this waste to land application will be dependent on the composition of organic waste constituents and the concentration of sulfuric acid. The MEK waste was used in the acute toxicity study, and only a limited characterization in the bioassays was performed.

The bio-solids waste from the production of phenol should contain acetophenone, phenol, cumyl phenol, and tars (EPA, 1980). The U.S. production of phenol in 1978 was greater than one billion kilograms. The phenol waste was collected in-line at a petrochemical plant using equipment provided by plant personnel. This waste is a liquid waste containing only 0.2% extractable hydrocarbons. Because of the low level of organic waste constituents and the physical state, this waste was used exclusively in the acute toxicity study.

The fourth petrochemical waste collected for the present study was the bio-solids from an agricultural chemical plant. The bio-solids waste was collected from a concrete wastewater treatment pit using equipment supplied by plant personnel. This waste contains the combined waste streams from a large agricultural chemical plant and may contain a variety of hazardous constituents. The bio-solids waste from the agricultural chemical plant was only used in the acute toxicity study because it was a liquid waste with a 0.2% extractable hydrocarbon content.

Three additional wastes were collected but were not used in the current study. These include the primary clarifier from a pulp and paper mill, an alum sludge, and a latex paint sludge. None of these wastes contained sufficient extractable hydrocarbons to merit any further evaluation.

Extraction

Two procedures were used for the extraction of hydrocarbons from wastes and waste-amended soils. The majority of samples were extracted using the blender technique, and comparisons were made with a limited number of samples using a Soxhlet extractor.

Dichloromethane was selected from a group of agents to extract the organic fractions of the wastes and the soil; dichloromethane consistently provided the greatest extraction efficiency for the type of materials anticipated (McGill and Rowell, 1980). Six volumes of dichloromethane were added to approximately 25 g of the waste or waste-soil mixture and mixed in a Waring blender for thirty seconds. This extraction was repeated twice or until the extracting solvent remained colorless. Solvent extractions were then combined and taken to dryness on a Brinkman Bucci Rotary Evaporator. The residue from this extraction was partitioned into acid, base, and neutral fractions following the scheme outlined in Figure 12. The neutral fraction of each waste was further separated into four subfractions using sequential

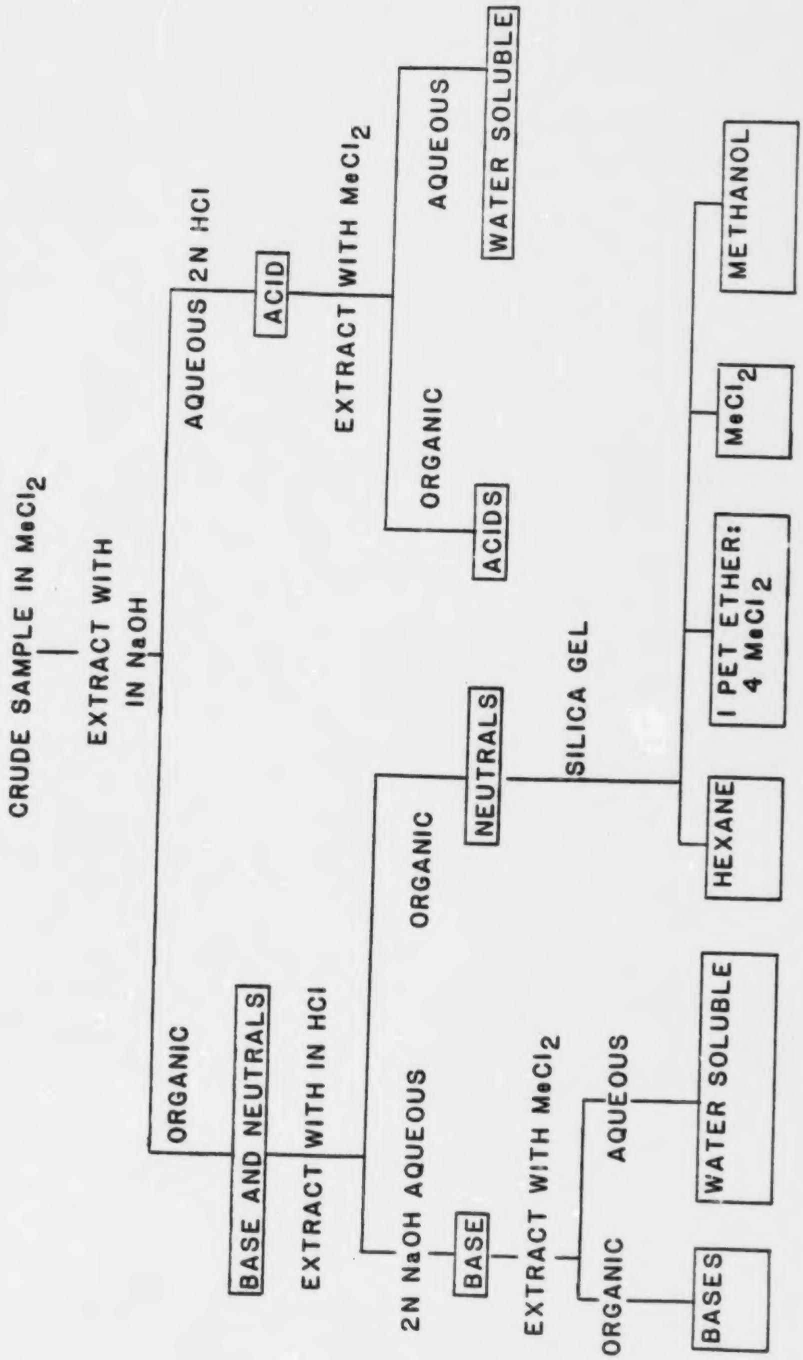
CRUDE SAMPLE IN $MeCl_2$
EXTRACT WITH IN NaOH
ORGANIC
BASE AND NEUTRALS
EXTRACT WITH IN HCl
2N NaOH AQUEOUS
BASE
EXTRACT WITH $MeCl_2$
ORGANIC
BASES
AQUEOUS
WATER SOLUBLE
ORGANIC
NEUTRALS
SILICA GEL
HEXANE
PET ETHER: 4 $MeCl_2$
$MeCl_2$
METHANOL
AQUEOUS 2N HCl
ACID
EXTRACT WITH $MeCl_2$
ORGANIC
ACIDS
AQUEOUS
WATER SOLUBLE

Figure 12. Fractionation scheme used for waste and waste-amended soils.

solvent extraction on a silica gel column. This extraction approximately separated the neutral fraction into saturate, aromatic, and condensed ring fractions according to the procedures of Warner *et al*. (1976).

A limited number of waste and waste-soil samples were also extracted using a Soxhlet apparatus to compare the efficiency of the two procedures. Fifteen to twenty grams of the homogenized waste or waste-soil mixture were weighed into a cellulose extraction thimble and mixed with a glass rod. The thimble and glass rod were placed in a 20 ml capacity Soxhlet extractor. Condensers and boiling flasks containing 200 ml of dichloromethane were assembled with the extractor and placed on the heater apparatus. Refrigerated coolant was circulated through the condensers. A 30 ml volume of solvent was introduced into the extractor from the top to facilitate flushing of the extractor chamber. Heat was applied to the boiling flask to initiate a 6 hour solvent extraction of the organic components; the temperature was adjusted to give six flushings per hour. The solvent extracts were then combined, taken to dryness, and partitioned according to the same procedures as used for the blender extracts.

Chemical Analysis

A chemical analysis of waste and soil-waste extracts was conducted by the USEPA's RS Kerr Environmental Research Laboratory. The compounds were identified using a Finnigan OWA Automated GC/MS. The GC capillary column used was a J&W Scientific DB-5-30W. One µl aliquots were used with a helium carrier gas flow near 36 cm/sec. The GC oven temperature program was 60°C for 1 minute and then increased at 6°/min to 260°C with a hold time of 12 minutes. The OWA unit had a splitless mode injector. The software used for analysis had a mass spectra library of 31,331 organic compounds.

Biological Analysis

Acute toxicity was determined for the crude extract of all ten wastes using at least one strain in each of the microbial bioassays. Overnight cultures of the appropriate strain were grown to a cell density of approximately 1×10^9 cells per ml and serially diluted from 10^{-1} through 10^{-6}. To 2.0 ml of top agar, 0.1 ml of the microbial culture and 0.1 ml of the crude extract of each waste were added, mixed on a vortex mixer, and plated on a complete medium. Cells were exposed to a minimum of four dose levels of the crude extract. The plates were incubated for 24 hours at 37°C, and fractional survival (N/N_o) was determined by comparing cell counts on exposed plates to the cell count on plates exposed to the solvent DMSO without the waste extract. Three of the 10 wastes were then selected for characterization of the chronic toxic effects and for use in the greenhouse and lysimeter studies.

The ability of samples to induce genetic damage was measured in three microbial systems (Table 14). The eukaryotic bioassay which employs *Aspergillus nidulans* (a fungus) can be used to detect point mutations and small deletions induced in a haploid genome or can be used as a diploid organism to detect chromosome aberrations, mitotic recombination, gene mutation, non-disjunction, recombinogenic events, recessive lethals, and spindle poisons. These systems are capable of detecting changes in the genetic entity that are of relevance to the human species and are sensitive to compounds not detected in the *Salmonella* assay (Lilly, 1965; Scott *et al*., 1978; Scott *et al*., 1982). In the first phase of this study, the *Aspergillus* bioassay will be used to assess the mutagenic potential of the acid, base, and neutral fractions of hazardous industrial wastes by evaluating the induction of mutations at the methionine suppressor loci. Conidia from 4 to 5 single colonies of the *methG1 biA1* (requiring methionine and biotin) Glasgow strain of *Aspergillus nidulans* were grown for 5 to 6 days on a complete medium at 37°C and were used for each experiment. Samples were tested at a minimum of three dose levels and four exposure times. The procedures used were the same as Scott *et al*. (1978). Mutant colonies were assayed by spreading exposed cells on a methionine free medium. Mutant colonies were scored after incubation for 5 days at 37°C. Colonies were divided by colony morphology into three classes, i.e., A, B, C, and the total number of mutant colonies. Each of these three classes is believed to involve two genes. The morphology of Class A colonies appear green, Class B brown, and Class C green with a white hyaline edge. The frequency of mutations induced by a sample was determined by subtracting the frequency of spontaneous mutations which occurred in Classes A, B, C, or the total from the total mutation frequency in Classes A, B, C and the total. A sample was considered mutagenic if there was a positive slope on the mutation induction curve, or the induced mutation frequency for at least two exposure times was more than twice the spontaneous mutation frequency. Positive controls included 8-methoxypsorlen (Sigma, St. Louis, MO), 8-methoxypsorlen plus near UV light without activation, and benzo(a)pyrene (Aldrich, Milwaukee, WI) with metabolic activation. The three waste fractions were also tested in a diploid bioassay. Diploid 20 (Kafer *et al*., 1982) was used to test the ability of waste fractions to induce mitotic crossing over, mitotic non-disjunction, breakdown of mitosis, and mutations with deleterious dominant effects. The procedures used were essentially the same as were used with the methionine system and are described in Section 4.

A microbial DNA repair assay was used to measure the capacity of a sample to produce increased lethal damage in DNA repair deficient strains. Six strains of *B subtilis* deficient in different recombination (Rec$^-$) and/or excision (Exc$^-$) repair were used to test for lethal DNA damage. These included the Rec$^-$ strains *rec A8*, *rec B2*, *rec E4*, *mc-1*; Exc$^-$ strain *hcr-9*; and Rec$^-$/Exc$^-$ *fh 2006-7*. All of these strains are isogenic with *B. subtilis* strain *168* which has all repair intact. These strains were kindly

TABLE 14. BIOLOGICAL SYSTEMS USED TO DETECT GENOTOXIC COMPOUNDS IN ENVIRONMENTAL SAMPLES

Organism	Genetic Event Detected	Advantages	Disadvantages	References
PROKARYOTES				
Bacillus subtilis	Increased lethal damage in DNA	Sensitive to bacteriocides, inorganics; can evaluate several DNA repair mechanisms;	Insensitive to volatiles; difficult to quantify.	Felkner *et al*., (1979). Kada *et al*., (1974). Tanooka *et al*.,(1978).
Salmonella typhimurium	Point mutation	Well-validated; well defined; end-point.	Insensitive to volatiles; toxic mutagens, certain chlorinated hydrocarbons.	Ames *et al*.,(1975). Skopek *et al*.,(1978). Haroun and Ames, (1981).
EUKARYOTES				
Aspergillus nidulans	Point mutation; chromosome damage.	Detects range of genetic damage including terratogens; good correlation in compounds	Limited data base; insufficient number of trained personnel.	Scott *et al*., (1982). Scott *et al*., (1978). Bignami *et al*., (1891).

supplied by Dr. I. C. Felkner of Clements Assoc., Washington, D.C. Overnight cultures were grown in brain-heart infusion broth (Difco, Detroit, MI) incubated at 37°C. Each strain was streaked radially on a nutrient agar plate to a centrally placed sensitivity disc containing 100 µl of the test chemical. After incubation at 37°C for 18 hours, the distance of growth inhibition from the disc was measured in millimeters (Kada _et al_., 1974). A response was considered positive if the distance of growth inhibition was more than 2.5 mm greater in one of the repair deficient strains than in the repair proficient strain _168_. Mitomycin C (Sigma), methylmethane sulphonate (Aldrich), and sensitivity to ultraviolet light were used as appropriate positive controls. Quadruplicate plates were run at each dose level for all samples.

Fractional survival (N/N_o) was determined for those strains showing the greatest sensitivity (inhibition) to the test chemical using the procedures of Donnelly _et al_. (1983). Brain heart infusion broth was inoculated with the appropriate strain and incubated at 37°C for approximately 16 h until an OD_{540} of 0.3 was reached (approximately 1.2×10^9 cells per ml). The cells were then diluted with brain heart infusion media to an OD_{540} of 0.16 (approximately 1.2 $\times 10^8$ cells per ml), serially diluted with Spizizen's Minimal Salts from 10^{-1} through 10^{-6}, and plated onto nutrient agar (Felkner _et al_., 1979).

The _Salmonella_/microsome assay of Ames _et al_. (1975) was used to evaluate the mutagenic activity of waste fraction samples. The _Salmonella_ strains were kindly supplied by Dr. Bruce N. Ames (University of California, Berkeley, CA). The procedural methods were the same as Ames _et al_. (1975) except that overnight cultures were prepared by inoculation into 10 ml of Oxoid Nutrient Broth #2 (KC Biological, Inc., Lenexa, KS) and incubated with shaking for 16 hours at 37°C. Waste fractions were tested on duplicate plates in two independent experiments in the standard plate incorporation assay at a minimum of 5 dose levels of the sample with and without enzyme activation (0.3 ml rat liver/ml S-9 mix) using strains TA98 and TA100. Positive controls included 2µg/plate N-methyl-N'-nitro-N-nitrosoguanidine (Sigma) for TA100, 25 µg/plate 2-nitrofluorene (Aldrich) for TA98, and 10 µg/plate 2-aminoanthracene (Sigma) which was used with all strains to verify the functioning of the metabolic activation system. All reagents and extracts were tested for sterility; DMSO was used as a negative control.

The sensitivity of in vitro bioassays can be greatly enhanced by incorporating mammalian metabolism into the testing protocol (Ames _et al_., 1973). This metabolic activation system serves to convert promutagens into their ultimate mutagenic form. Metabolic activation can be incorporated into the testing protocol by the addition of an S9 mix which consists of a 9,000 x G supernatent from homogenized rat liver and an NADPH generating system. Rat liver was utilized as the source of enzymes because studies conducted to evaluate the activity of S9 liver fractions from various species concluded that rat liver provides activation for the broadest range of chemicals (Baker et al., 1980; Muller et al., 1980). A 50 ml batch of S9 mix consists of 85 mg glucose-6-phosphate (Sigma), 160 mg NADP (Sigma), 10 ml of 0.5 m phosphate buffer, 25 ml cation solution, and 15 ml of S9 (Matney et al., 1979). The majority of testing was conducted using Aroclor 1254 induced rat liver which is considered to activate the broadest range of chemicals (Maron and Ames, 1983). In addition, a limited number of fractions were also tested using phenobarbitol induced S9 which is considered to work more efficiently with the basic fraction (Epler et al., 1978). Both Aroclor 1254 and Phenobarbitol induced rat liver were obtained from Litton Bionetics (Charleston, SC). The concentration of rat liver in the S9 mix is critical for obtaining the optimum response and will be variable from one compound to another (Maron and Ames, 1983). A limited number of samples were tested at three concentrations of S9/plate to determine the optimum concentrations for the types of mixtures being studied. The standard test was run at 0.3 ml rat liver/ml S9 mix with additional testing conducted at 0.5 and 0.1 ml rat liver/ml S9 mix.

RESULTS AND DISCUSSION

The acute toxicity of the crude extract of ten hazardous wastes was determined in bioassays using A. nidulans, B. subtilis, and S. typhimurium. The results for eight of the ten wastes are provided in Figure 13a-d and Figure 14a-d. These studies were conducted in order to establish maximum dose levels for the chronic toxicity study and to determine the potential short-term effects of waste application on bacteria and fungi. The acute toxicity of the bio-solids wastes from the production of phenol and from the agricultural chemical plant are not included as these wastes had very low organic content and almost no acute toxic effects in the bioassays at the levels tested. For most of the wastes studied, the acute toxic effects were greatest in prokaryotic Salmonella and Bacillus, and least in eukaryotic diploid Aspergillus. Only the crude extract of the methyl ethyl ketone waste (Figure 14b) was more toxic in the eukaryotic system than in the systems using prokaryotic organisms. For the Aspergillus system, only the wood-preserving liquid waste (Figure 13c) exhibited a definite increase in toxicity with increasing dose of the waste, while the slop-oil emulsion solids exhibited a slight increase in toxicity with increasing dose. For the Salmonella and Bacillus systems, all waste extracts studied produced an increase in toxicity with increasing dose. At least one dose level was determined for each waste at which less than 10% of the exposed prokaryotic cells survived. This dose will be used as the maximum dose level for all subsequent testing with the waste as well as with soil or water extract.

The mutagenic potential of the fractions of five hazardous wastes was evaluated in the battery of bioassays. The results of the

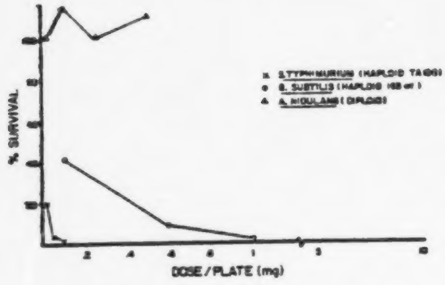

Figure 13a. Percent survival of B. subtilis strain 168, S. typhimurium strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium after exposure to various concentrations of wood-preserving bottom sediments waste. The percent survival (N/No x 100%) was then determined.

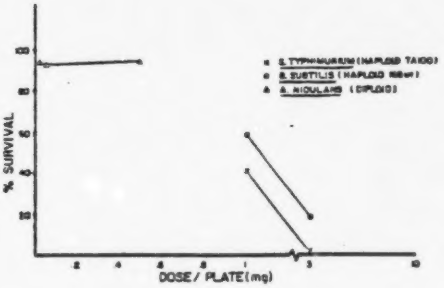

Figure 13b. Percent survival of B. subtilis strain 168, S. typhimurium strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium acetonitrile purification column waste. The percent survival (N/No x 100%) was then determined.

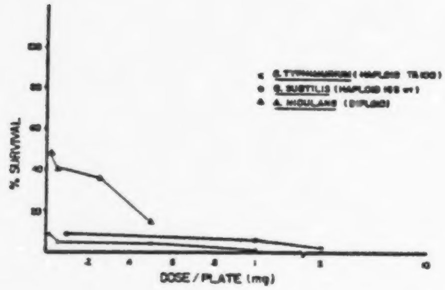

Figure 13c. Percent survival of B. subtilis strain 168, S. typhimurium strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium after exposure to various concentration of wood-preserving liquid waste. The percent survival (N/No x 100%) was then determined.

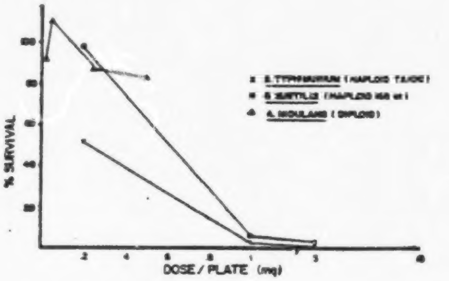

Figure 13d. Percent survival of B. subtilis strain 168, S. typhimurium strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium after exposure to various concentrations of slop-oil emulsion solids waste. The percent survival (N/No x 100%) was then determined.

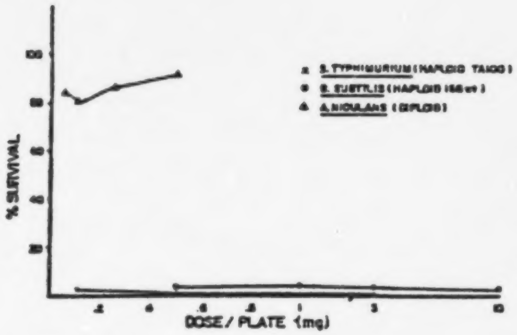

Figure 14a. Percent survival of B. subtilis strain 168, S. typhimurim strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium after exposure to various concentrations of dissolved air floatation float waste. The percent survival (N/No x 100%) was then determined.

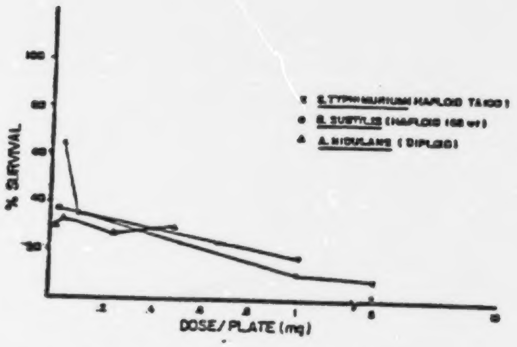

Figure 14b. Percent survival of B. subtilis strain 168, S. typhimurium strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium after exposure to various concentrations of methyl ethyl ketone waste. The percent survival (N/No x 100%) was then determined.

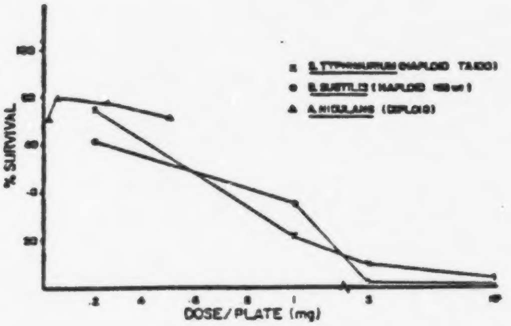

Figure 14c. Percent survival of B. subtilis strain 168, S. typhimurium strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium after exposure to various concentrations of storm-water runoff impoundment waste. The percent survival (N/No x 100%) was then determined.

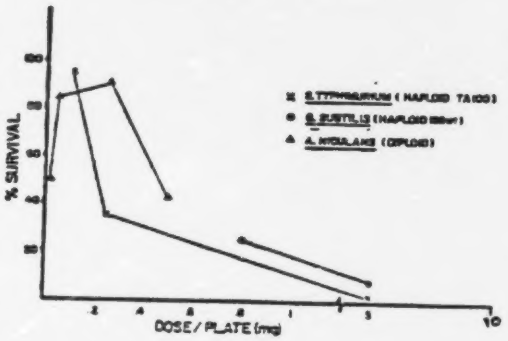

Figure 14d. Percent survival of B. subtilis strain 168, S. typhimurium strain TA100, and A. nidulans Diploid 109. The cells were plated on a complete medium after exposure to various concentrations of combined API separator/waste-water treatment sludge waste. The percent survival (N/No x 100%) was then determined.

Salmonella/microsome mutagenicity assay of the acetonitrile waste are presented in Figure 15 and Table 15. These results indicate that mutagenic activity could be detected in all three waste fractions and that primarily indirect acting mutagens were detected. The dose-response curves for all fractions were non-linear, indicating that the fractions are composed of constituents with non-equivalent kinetics of mutation induction. The base and neutral fractions induced a two-fold increase in revertant colonies in both strain TA98 and TA100, while the acid fraction only induced a significant increase in strain TA98. The basic fraction of the waste induced the greatest number of revertant colonies in both strains. At an exposure level equivalent to almost 11 g of the original waste material (5.0 mg/plate of extract), the basic fraction induced 328 and 366 revertant colonies in strain TA98 and TA100, respectively (Table 15). In the absence of metabolic activation, a doubling of revertant colonies was induced by all three fractions in TA98 at dose levels greater than 5 mg/plate. The addition of metabolic activation to the assay system resulted in an increase of at least twice the number of revertant colonies than was obtained without metabolic activation.

One of the known constituents of this waste, acetamide, induced less than 14 revertant colonies/mg at a dose level of 5 mg (McCann et al., 1975). At the same dose level, the acid, base, and neutral fractions induced 31, 71, and 21 net revertants/mg, respectively. Although the acetonitrile waste contained the experimental carcinogens acetamide (Sax, 1979), it also contained small concentrations of other mutagenic agents or agents that promoted the activity of acetamide in the various waste fractions.

An evaluation of the acid, base, and neutral fractions of the acetonitrile waste using the B. subtilis DNA repair assay (Table 16) indicates that none of the waste fractions induced increased lethal damage in the repair deficient strains. Two limitations of the DNA repair spot test are limited sensitivity to compounds which are indirect acting and limited sensitivity to compounds which are insoluble in water. The negative response in the DNA repair assay may have been due to the absence of metabolic activation in this test, since metabolic activation was required to obtain the maximum response in the Salmonella assay.

In conclusion, the determination of the mutagenic potential of the fractions of acetonitrile waste indicates that the fractions contain primarily indirect acting mutagens, and the base fraction was the most active. The base fraction induced 306 net revertants in the Salmonella assay at a dose level equivalent to approximately 11 g of waste. By comparison, the basic N-heterocyclic compound 10-azobenzo[a]pyrene induced 130,000 revertants/mg in an evaluation by Ho et al. (1981). Thus, the mutaganic potential of the components of the base fraction appears to be much lower than that of the substituted polycyclic aromatic hydrocarbon.

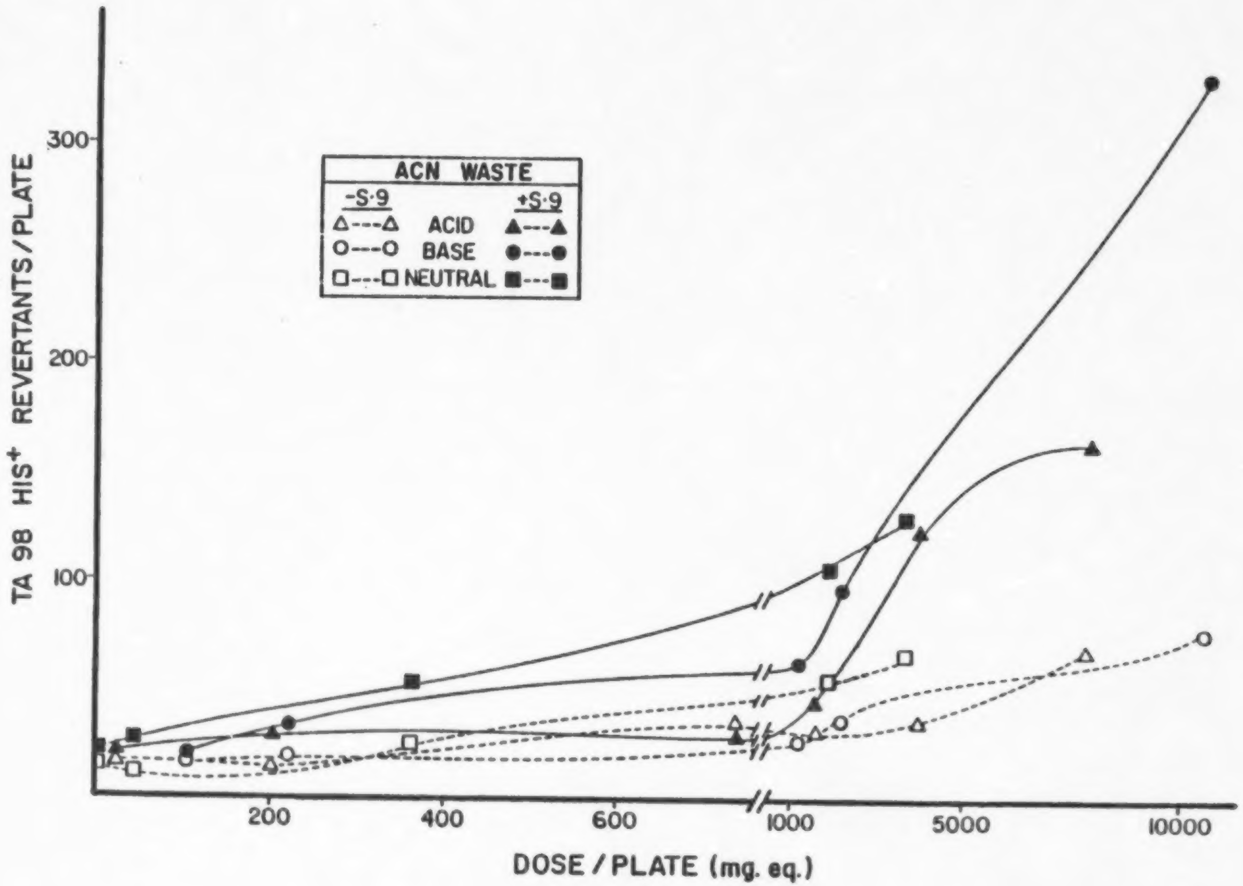

Figure 15. Mutagenicity, as measured with S. typhimurium strain TA98, of fractions of acetonitrile waste.

TABLE 15. MUTAGENIC ACTIVITY OF LIQUID STREAM FROM ACETONITRILE PURIFICATION COLUMN AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA98 AND TA100 WITH AND WITHOUT METABOLIC ACTIVATION

Sample	Dose Level (mg/plate)	Strain TA98 + S-9	Strain TA98 - S-9	Strain TA100 + S-9	Strain TA100 - S-9
		Total His$^+$ revertants/plate - Mean ± S.D.			
Acid	5.0	162 ± 31	69 ± 3	197 ± 31	188 ± 58
	2.5	122 ± 2	36 ± 5	NT*	149 ± 1
	1.0	45 ± 13	32 ± 5	145 ± 14	85 ± 11
	0.5	30 ± 5	36 ± 7	155 ± 3	149 ± 6
	0.1	30 ± 14	15 ± 4	140 ± 18	80 ± 9
	0.01	20 ± 4	17 ± 4	140 ± 24	80 ± 5
Base	5.0	328 ± 5	77 ± 12	366 ± 76	109 ± 5
	1.0	97 ± 32	36 ± 4	173 ± 4	112 ± 17
	0.5	62 ± 1	28 ± 2	143 ± 15	182 ± 12
	0.1	35 ± 15	20 ± 1	120 ± 18	92 ± 14
	0.01	20 ± 2	17 ± 0	140 ± 8	87 ± 15
Neutral	10.0	126 ± 10	67 ± 1	326 ± 47	131 ± 21
	5.0	106 ± 21	55 ± 2	289 ± 15	NT
	1.0	55 ± 5	27 ± 8	87 ± 34	102 ± 35
	0.1	36 ± 19	13 ± 1	97 ± 39	93 ± 20
	0.01	21 ± 3	15 ± 3	79 ± 25	85 ± 4
DMSO	100 µl	22 ± 5	19 ± 3	117 ± + 21	103 ± 164

* NT = Not tested.

TABLE 16. COMPARISON OF LETHAL EFFECTS OF ACN WASTE FRACTIONS ON DNA REPAIR DEFICIENT AND PROFICIENT STRAINS OF B. SUBTILIS

Sample	Dose/pt	Inhibition Radius (mm)						
		168 wt RP[1]	fh2006.7	recE4	mc-1	hcr.9	recA8	recB2
			repair deficient					
Acid	1 mg	0	1	0	1	0	0	0
Base	1 mg	0	1	1	0	2	1	1
Neutral	10 mg	0	0	0	1	0	0	0
MMS[2]	2 µl	14	26	25	20	21	21	18
Mit.C[3]	10 µg	6	11	11	12	13	13	13
DMSO[4]	100 µl	0	0	0	0	0	0	0

1- Repair Proficient
2- Methyl methane sulfonate
3- Mitomycin C
4- Dimethylsulfoxide

The methyl ethyl ketone waste was found to be genotoxic in only one of the three bioassays. In the Aspergillus methionine system, a positive response was obtained with the neutral fraction (Figure 16 and Table 17). These results indicate that for colony A, B, C and the total, there was a significant increase in mutant colonies. The results from the Aspergillus bioassay are in contrast with those from the Bacillus and Salmonella assays (Figures 17 and 18 and Table 18). The DNA repair deficient strain mc-1 gave a weak positive response when exposed to 2.5 mg of the acid fraction, while no response was obtained in any of the other strains with the base and neutral fractions. The acid and neutral fractions induced a doubling of revertant colonies in S. typhimurium strain TA98 with metabolic activation only at the highest tested dose level. Using the modified 2-fold rule described by Chu et al. (1981), this would not be considered a positive response. Biological analysis of the methyl ethyl ketone waste disclosed the presence of marginal genetic toxicity. The neutral fraction was positive in Aspergillus and negative in both Salmonella and Bacillus; however, the acid and base fractions were negative in all three bioassays.

The most intensive analysis was conducted on the wood-preserving bottom sediment (PENT S). Samples of the PENT S waste were collected on two separate occasions and returned to the laboratory for fractionation and characterization. A subsample of the second sample collected was also extracted using the Soxhlet apparatus and evaluated using Salmonella strain TA98. The first waste sample collected was used in the greenhouse study, while the second sample was used in the lysimeter study. The results presented in Table 19 indicate that although greater amounts of extractable hydrocarbons were recovered using the Soxhlet apparatus, there was no appreciable difference in the specific activity of the fractions. The similarity of the mutagenic activity obtained from the three extractions is most evident when the dose response curves for the crude, acid, base, and neutral fractions are compared (Figures 19a-d). At lower dose levels, the mutagenic responses obtained from each of the three extractions were within 10% of each other. At the two highest dose levels tested, the mutagenic activity of the crude, base, and neutral fractions of the Soxhlet extraction was lower than the other two extractions by 20 to 50%. These results indicate that greater quantities of hydrocarbons can be extracted from the soil using a Soxhlet apparatus than with the Blender procedure. Both procedures did, however, give approximately equivalent responses when tested in the Salmonella/microsome assay, indicating that the composition of mutagenic compounds in the extracts was similar.

The results from biological analysis of the PENT S fractions using four strains of S. typhimurium are provided in Table 20. A positive response was obtained from the crude, acid, base, and neutral fractions of the PENT S waste with strains TA98, TA100, and TA1538 in the presence of metabolic activation. None of the waste fractions induced a positive response in the absence of metabolic activation, and none induced a

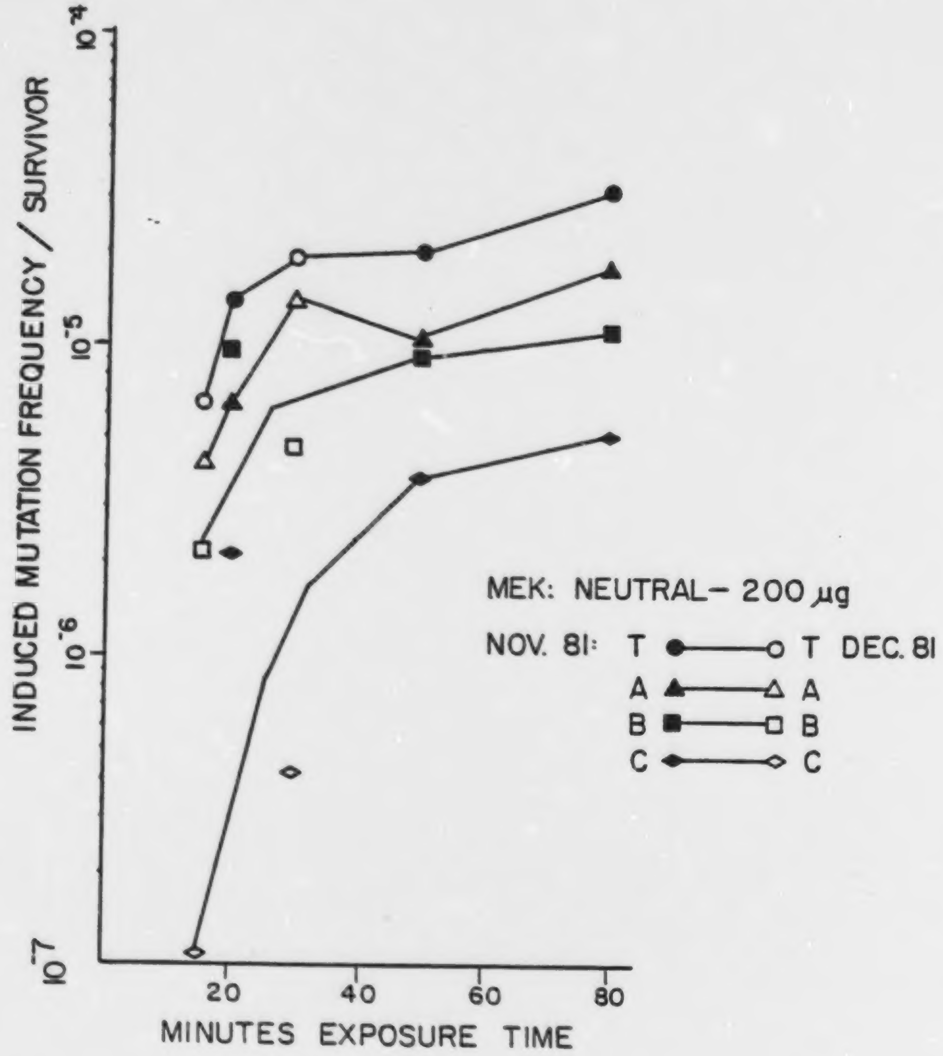

Figure 16. Mutation frequency induced by neutral fraction of methyl ethyl ketone (MEK) waste in *A. nidulans* methionine system.

TABLE 17. THE EFFECT OF THE NEUTRAL FRACTION OF A METHYL ETHYL KETONE WASTE ON THE FREQUENCY OF INDUCED MUTATIONS IN *ASPERGILLUS NIDULANS*

Exposure				Mutation Frequency Per 10^6 Survivors			
Dose (mg/pl)	Time (min)	Number of Cells Plated	Surviving Fraction	A	B	C	Total
0.2	0	2.4×10^7	1.0	-	-	-	-
0.2	15	2.8×10^7	0.89	4.27	2.27	.076	6.61
0.2	20	1.4×10^7	0.83	6.19	9.67	.222	16.1
0.2	30	1.7×10^7	0.55	14.4	4.80	.409	19.6
0.2	45	1.3×10^7	0.72	10.6	8.9	.393	19.6
0.2	80	0.9×10^7	0.52	17.1	11.1	2.4	30.6

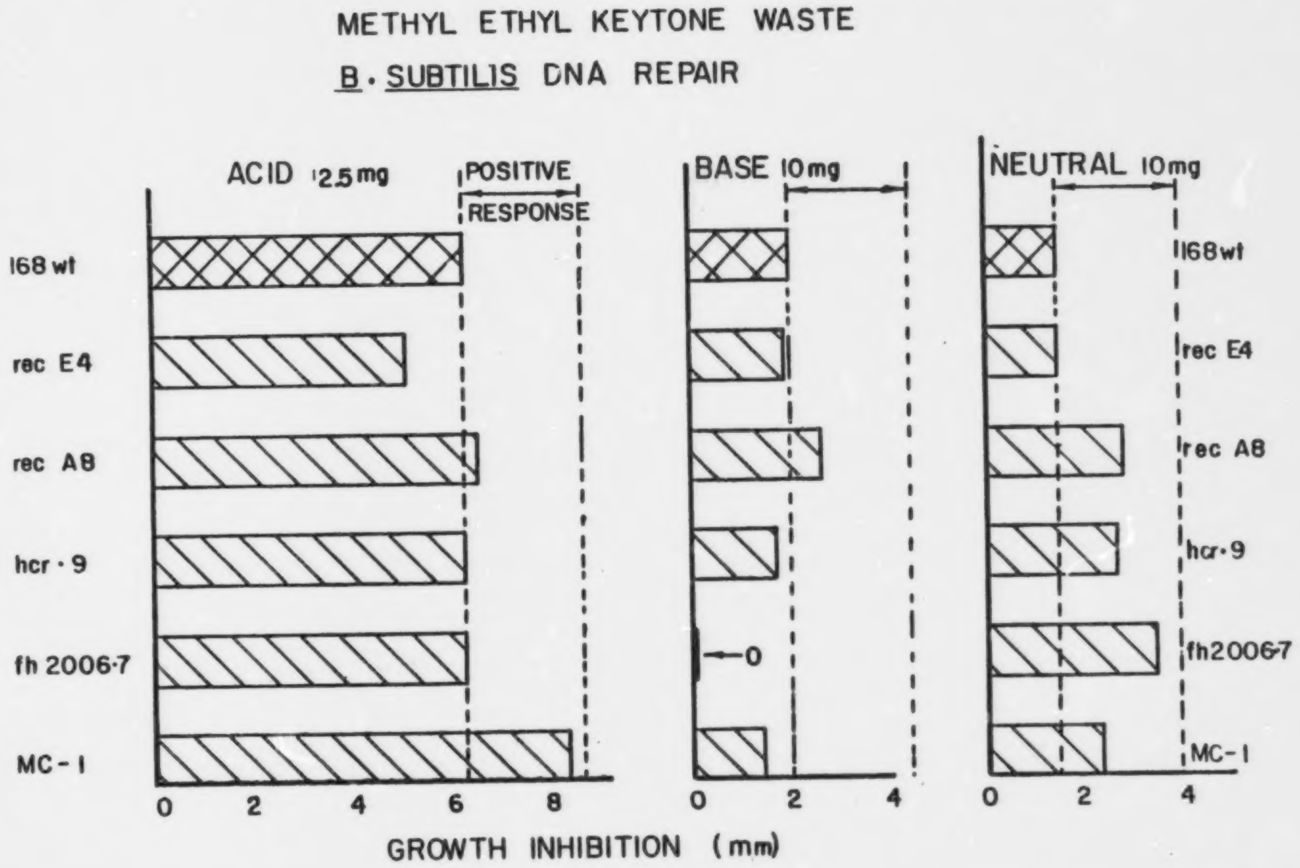

Figure 17. Growth inhibition induced by fractions of methyl ethyl ketone waste in DNA repair proficient (168 wt) and deficient strains of B. subtilis.

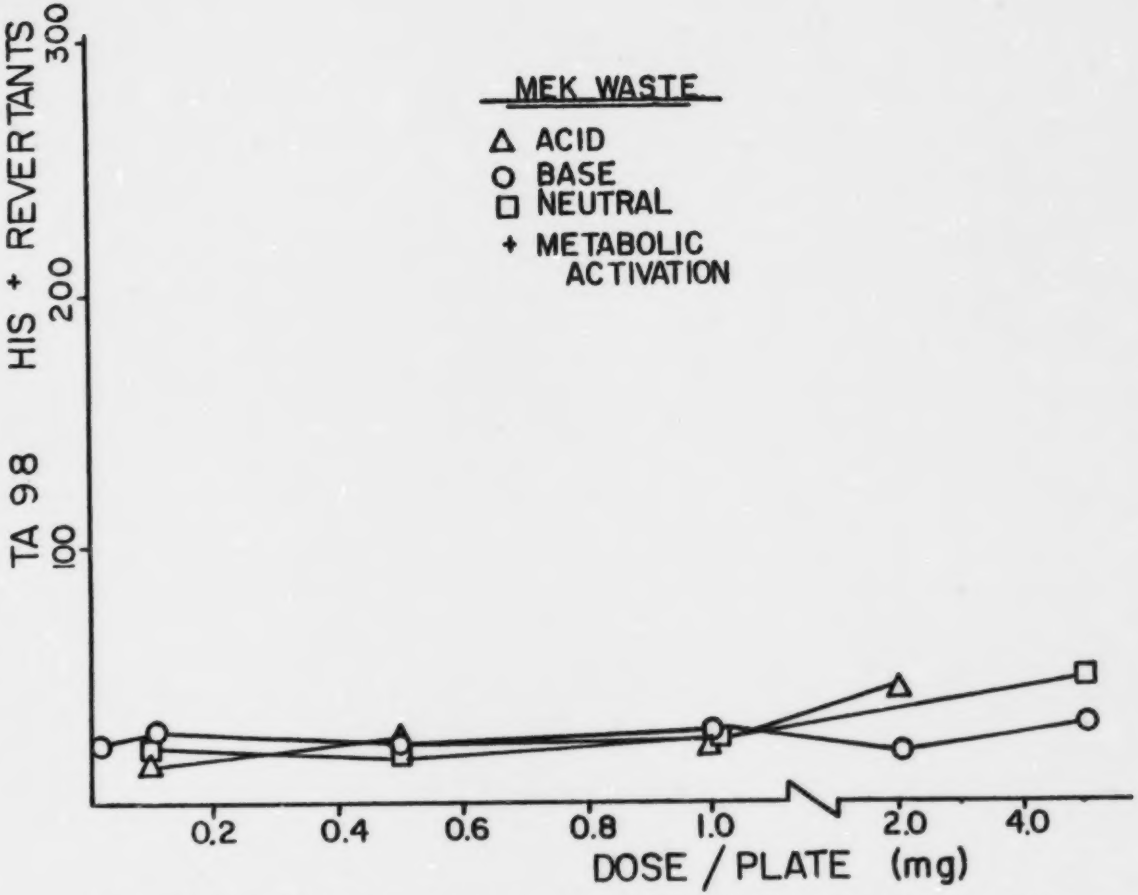

Figure 18. Mutagenic activity, as measured in *S. typhimurium* strain TA98, of fractions of methyl ethyl ketone waste (MEK).

TABLE 18. MUTAGENIC ACTIVITY OF METHYL ETHYL KETONE WASTE

Sample	Dose Level (mg/plate)	Strain TA98 + S-9	Strain TA98 - S-9	Strain TA100 + S-9	Strain TA100 - S-9
		Revertant Colonies per plate + S.D.			
Acid	2.5	43 ± 3*	30 ± 9	NT**	77 ± 9
	1.0	22 ± 2	22 ± 7	NT	70 ± 1
	0.5	29	27 ± 9	NT	80 ± 2
	0.1	14 ± 1	40 ± 23	NT	74 ± 0
Base	5.0	33 ± 2	36 ± 4	65 ± 3	94 ± 5
	2.5	18 ± 1	NT	NT	84 ± 5
	1.0	24 ± 1	21 ± 6	78 ± 1	75 ± 5
	0.5	28 ± 1	18 ± 1	NT	81 ± 1
	0.1	29 ± 1	17 ± 2	70 ± 2	80 ± 2
	0.01	22 ± 2	17 ± 4	NT	
Neutral	5.0	49 ± 6*	20 ± 6	100 ± 14	110 ± 16
	1.0	25 ± 8	21 ± 7	79 ± 5	67 ± 2
	0.5	24 ± 1	18 ± 2	NT	73 ± 4
	0.1	24 ± 8	25 ± 4	NT	86 ± 2

* - significant increase.

**- NT = Not tested.

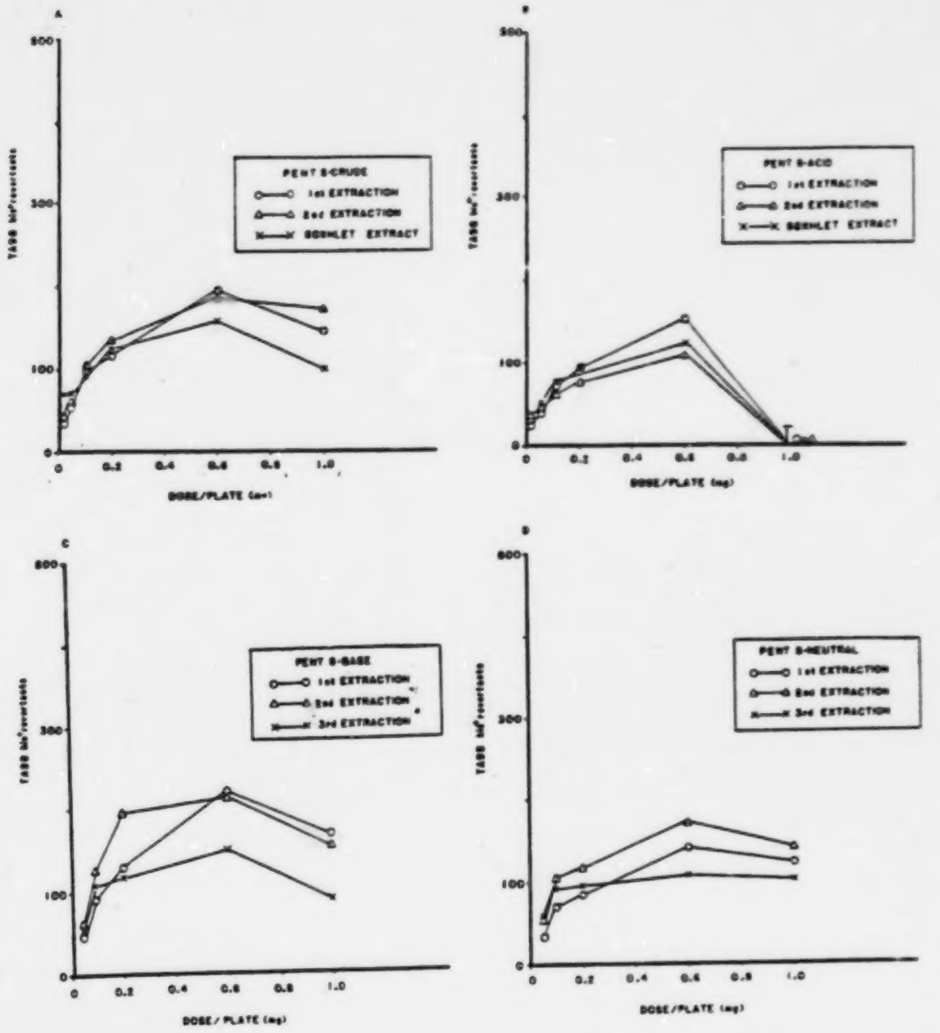

Figure 19. Mutagenic activity, with metabolic activation, of crude (A), acid (B), base (C), and neutral (D) fraction of PENT S (wood preserving bottom sediment) from two blender extractions and one soxhlet extraction.

TABLE 19. DISTRIBUTION OF MUTAGENIC ACTIVITY[1] IN FRACTIONS OF PENT S WASTE EXTRACTED USING BLENDER OR SOXHLET TECHNIQUE

Sample	Extractable Hydrocarbon (mg/g)	Specific Activity[2] (rev/mg)	Weighted Activity[3] (rev/g)
Blender (1)			
Crude	270	1,282	346
Acid	23	771	18
Base	24	1,204	29
Neutral	223	860	192
Blender (2)			
Crude	271	1,154	313
Acid	9	828	7
Base	3	1,657	5
Neutral	259	1,186	307
Soxhlet			
Crude	590	1,162	686
Acid	21	904	19
Base	8	1,209	10
Neutral	561	1,036	581

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1. Mutagenic activity as measured with strain TA98 with microsomal activation.
2. Revertants/mg = slope of mutation induction curve calculated using three highest non-toxic dose levels.
3. Revertants/gram eq = revertants/gram material extracted; calculated by multiplying revertants/mg x % extractable hydrocarbons.

TABLE 20. MUTAGENIC ACTIVITY OF FRACTIONS OF WOOD-PRESERVING BOTTOM SEDIMENT

Sample	Dose/Plate (mg)	Total his^+ revertants							
		TA98		TA100		TA1535		TA1538	
		-	+	-	+	-	+	-	+
Crude	0	25	42	144	133	NT	NT	NT	NT
	1	17	172	48	243				
	.5	31	183	111	521				
	.1	30	132	145	466				
	.05	27	108	149	377				
	.01	21	61	147	187				
	.005	23	44	126	155				
Acid	0	27	41	133	139	25	17	13	17
	1	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
	.5	26	111	Tox	403	Tox	13	Tox	Tox
	.1	24	77	108	331	11	15	10	58
	.05	25	66	112	247	24	15	8	36
	.01	26	48	122	159	18	16	13	19
	.005	24	37	109	149	15	17	NT	15
Base	0	26	41	116	139	25	17	11	16
	1	26	167	72	415	19	15	16	60
	.5	25	222	88	563	18	15	15	38
	.1	24	131	121	543	15	16	10	37
	.05	27	93	103	408	15	17	15	32
	.01	27	48	104	175	14	17	8	32
Neutral	0	21	33	121	133	25	17	13	17
	1	28	123	101	328	18	15	19	47
	.5	23	140	91	391	15	16	19	17
	.1	23	86	103	527	14	10	20	33
	.05	28	72	96	209	16	15	19	24
	.01	21	36	98	176	20	15	15	23

NT - not tested.

positive response in strain TA1535 with or without metabolic activation. The maximum response was obtained with the base fraction using strain TA98 with metabolic activation (Figure 20). At the optimum dose level of 500 µg/plate, the base fraction induced 222 revertant colonies, or greater than five times the background for strain TA98 (Table 20). At the same dose level, the base fraction induced 563 revertant colonies in strain TA100, or slightly greater than four times the background level (Figure 21). The mutagenic response in the two plasmid containing strains (TA98 and TA100) were comparable; however, the absence of a positive response in strain TA1535 indicates that constituents in the PENT S waste may be selective for frameshift mutants such as TA1538. A review of the literature by Wassom et al. (1977) observed that tetrachlorodibenzodioxin selectively induces frameshift mutations.

The sensitivity of the Salmonella/microsome assay has been further enhanced by the addition of two new plasmid carrying strains, TA97 and TA102 (Levin et al., 1982a; Levin et al., 1982b). Strain TA97 detects frameshift mutants and is intended to replace TA1537 (Levin et al., 1982a), while strain TA102 detects base-pair mutations and has been found to be sensitive to oxidative mutagens (Levin et al., 1982b). The results obtained from a biological analysis of the fractions of the PENT S waste using strain TA97 and TA102 were comparable to those obtained using strains TA98 and TA100 (Table 21).

Additional testing of the PENT S waste fractions was conducted with strain TA98 with high (0.5 ml S9/ml S9 mix), medium (0.3 ml S9/ml S9 mix), and low (0.1 ml S9/ml S9 mix) concentrations of liver microsomes. Maron and Ames (1983) recommend that these titrations be conducted as the concentration of S9 per plate is critical for optimum mutagenesis and can be variable from compound to compound. Rao et al. (1978) also observed that the concentration of microsomal enzymes can significantly affect the results of the biological testing of complex mixtures. The results presented in Table 22 and Figures 22, 23, and 24 indicate that maximum mutagenicity was detected with the high concentration of S9. There was, however, no appreciable difference between the level of mutagenic activity in the acid and base fractions with the high and medium concentrations of S9 (Figures 22 and 23). In the neutral fraction, the mutagenicity with the high concentration of S9 was significantly greater than the medium and low concentrations (Figure 24). However, for all three waste fractions, the optimum, toxic, and no-effect dose levels were the same with all concentrations of S9 mix. These results indicate that while the medium level of S9 in the S9 mix provides an adequate source of metabolic activation of most waste fractions, slightly greater levels of mutagenic activity may be observed in the presence of higher levels of S9.

A bioassay using B. subtilis was utilized to evaluate the capacity of wood-preserving bottom sediment fractions to produce increased lethal damage in DNA repair deficient bacteria. The acid

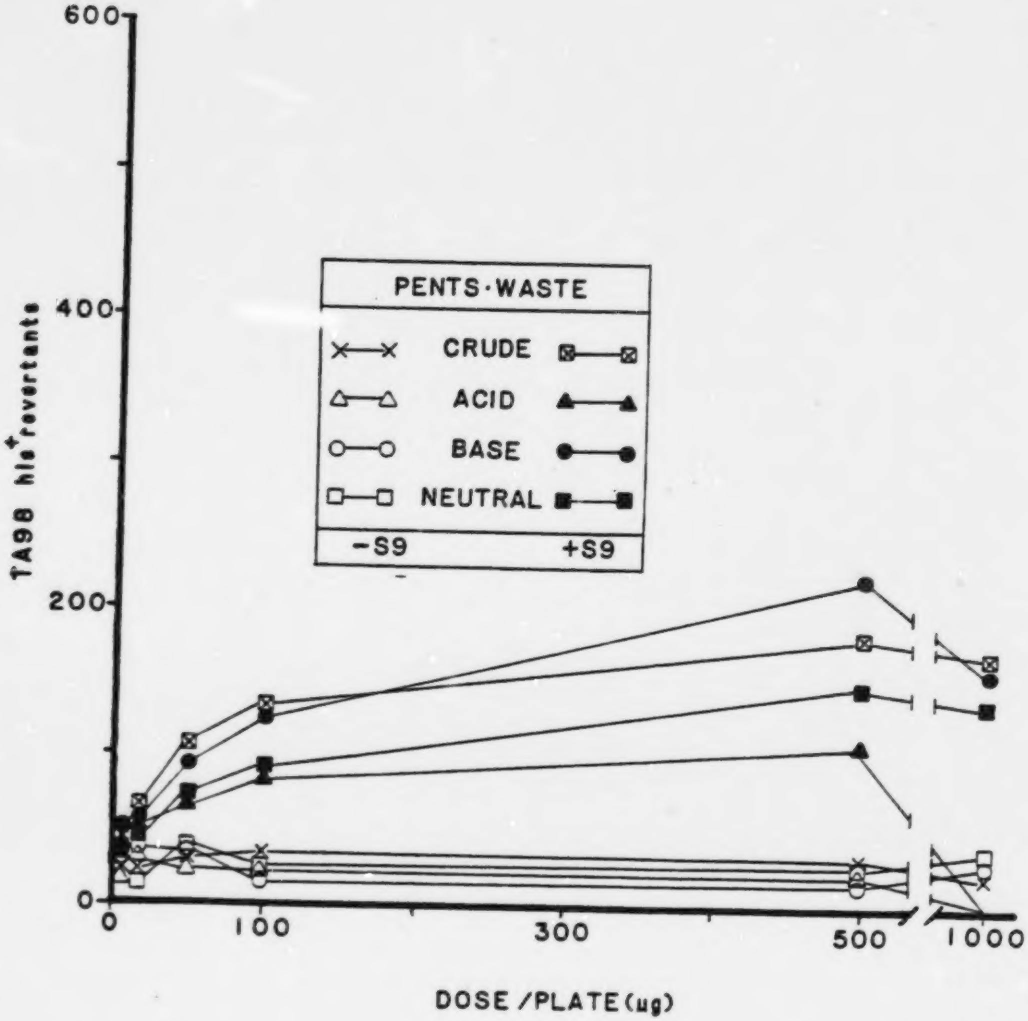


Figure 20. Mutagenic activity of fractions of the PENT S (wood preserving bottom sediment) waste.

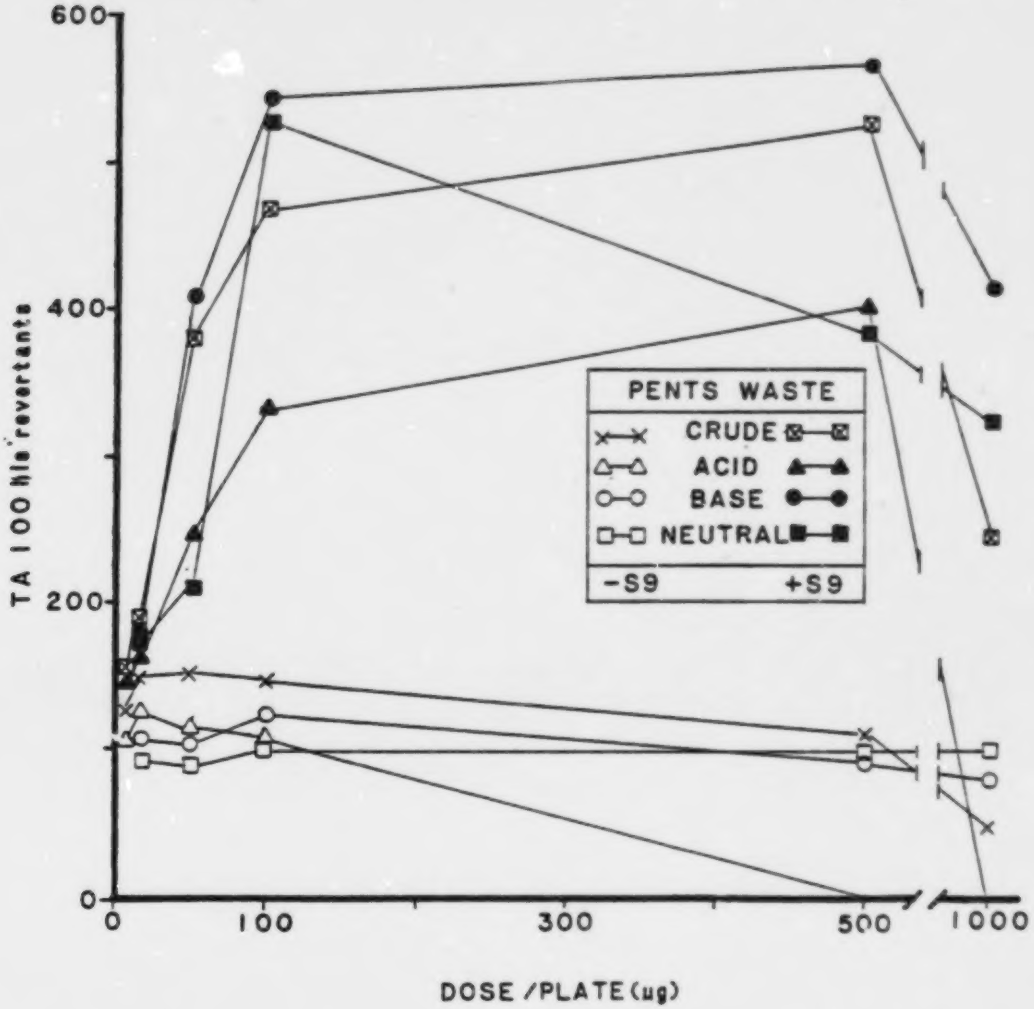


Figure 21. Mutagenic activity, as measured in S. typhimurium strain TA100, of fractions of PENT S (wood preserving bottom sediments) waste.

TABLE 21. MUTAGENIC ACTIVITY OF PENT S WASTE IN FOUR PLASMID CONTAINING STRAINS OF S TYPHIMURIUM

	Strain (± Metabolic Activation)							
Fraction	TA98		TA100		TA97		TA102	
	-	+	-	+	-	+	-	+
	net revertants/mg							
Acid	LB[1]	333	LB	1556	70	1419	LB	2300
Base	LB	463	16	1721	15	229	LB	7
Neutral	25	278	LB	1550	122	1975	LB	1186

1 - LB = Less than background.

TABLE 22. MUTAGENIC ACTIVITY OF PENT S WASTE FRACTIONS AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA98 WITH HIGH (0.5 ML S9/ML S9 MIX), MEDIUM (0.3 ML S9/ML S9 MIX) AND LOW (0.1 ML S9/ML S9 MIX) CONCENTRATIONS OF AROCLOR 1254 INDUCED RAT LIVER IN S9 MIX

Fraction	Dose/plate (mg)	Metabolic Activation		
		High	Medium	Low
		------------ Aroclor 1254	------------	------------
Acid	0	37 ± 7	41 ± 3	37 ± 6
	1	NT*	Tox	NT
	.5	126 ± 10	111 ± 17	79 ± 29
	.1	85 ± 14	77 ± 2	83 ± 3
	.05	53 ± 10	66 ± 13	53 ± 10
	.01	53 ± 5	48 ± 11	50 ± 10
Base	0	37 ± 7	41 ± 3	37 ± 6
	1	222 ± 51	167 ± 11	143 ± 62
	.5	226 ± 24	222 ± 18	162 ± 53
	.1	113 ± 17	131 ± 16	115 ± 19
	.05	95 ± 11	93 ± 17	79 ± 20
	.01	61 ± 23	48 ± 14	47 ± 9
Neutral	0	37 ± 7	33 ± 7	37 ± 6
	1	201 ± 17	123 ± 36	108 ± 46
	.5	210 ± 32	140 ± 29	135 ± 74
	.1	102 ± 12	86 ± 24	108 ± 12
	.05	88 ± 7	72 ± 15	104 ± 9
	.01	52 ± 8	34 ± 16	50 ± 6

* NT = Not tested.

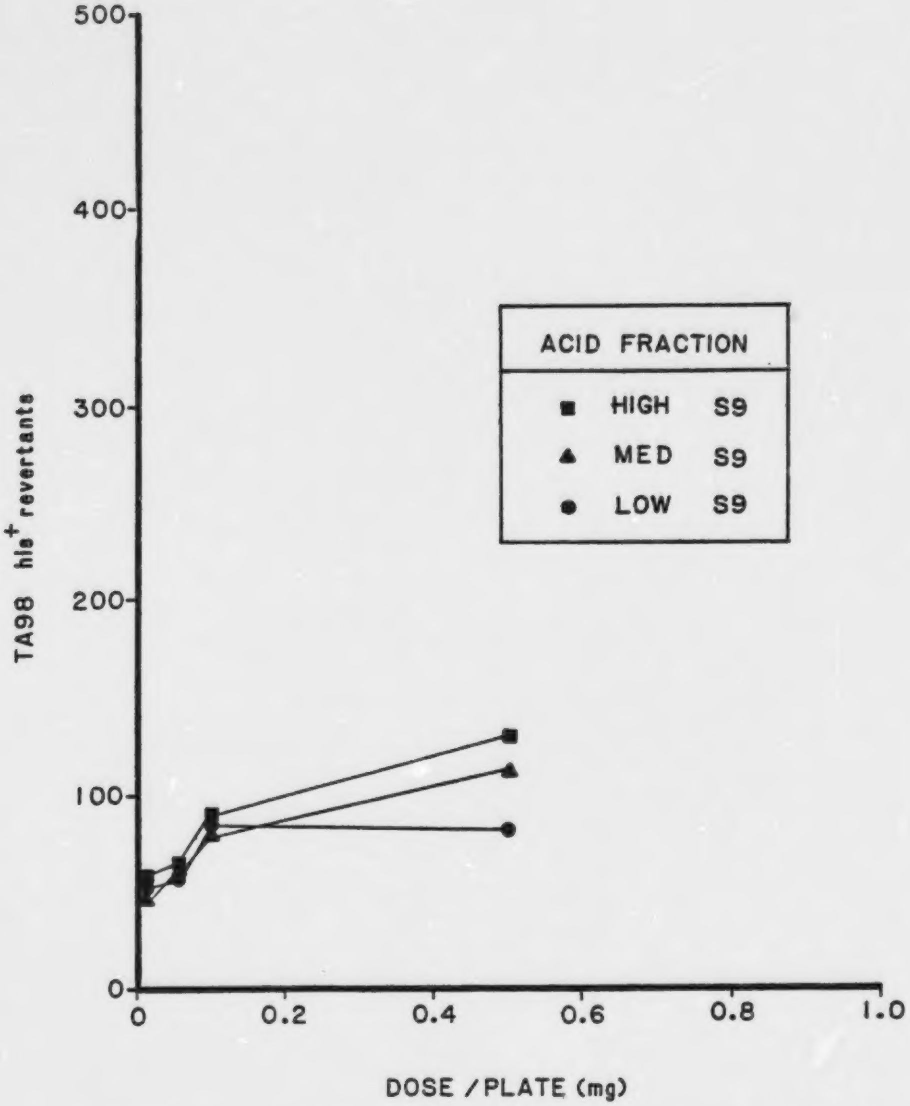


Figure 22. Mutagenic activity of acid fraction of PENT S waste as measured with high, medium, and low levels of S9 in the S9 mix.

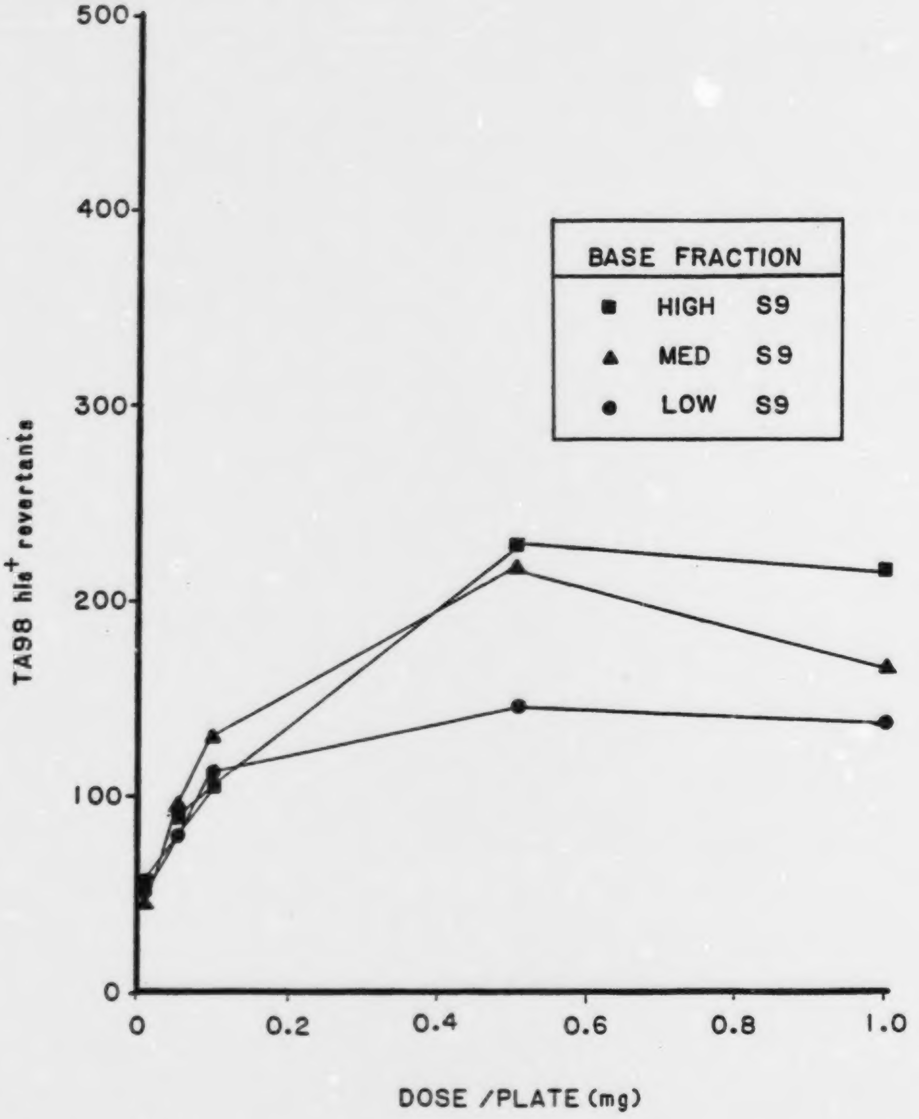


Figure 23. Mutagenic activity of base fraction of PENT S waste as measured with high, medium, and low levels of S9 in the S9 mix.

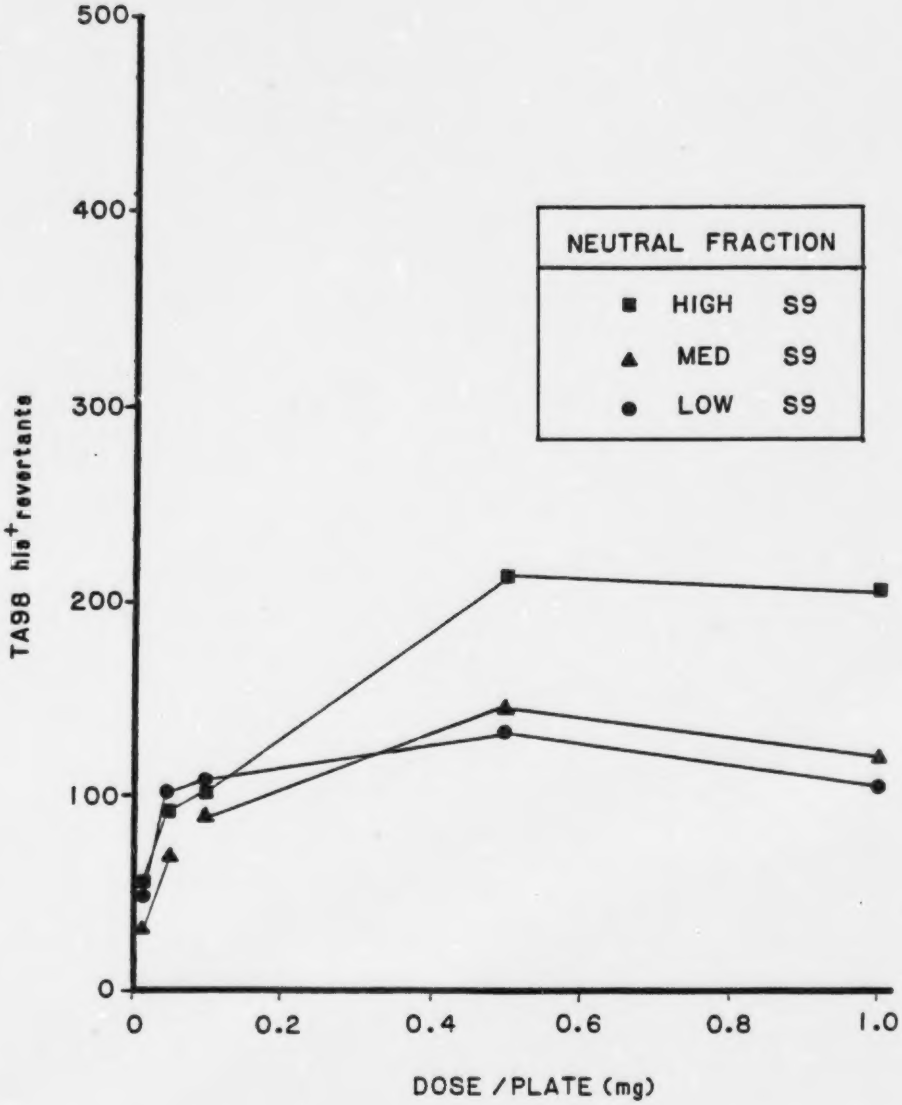


Figure 24. Mutagenic activity of neutral fraction of PENT S waste as measured with high, medium and low levels of S9 in the S9 mix.

fraction was the only fraction to induce a significant inhibition in the repair deficient strain when evaluated in the spot test (Table 23 and Figure 25). When tested in the modified plate incorporation assay, the base fraction also produced increased lethal damage in the repair deficient strains, while the response obtained from the neutral fraction was less conclusive (Table 24). The different responses obtained using the two procedures may have been a result of the lack of diffusion of waste fractions in the spot test. The modified plate incorporation assay did, however, conclusively demonstrate that the acid and base fractions of the PENT S waste contained constituents that are capable of producing increased lethal damage in repair deficient bacteria.

The capacity of PENT S fractions to induce genetic damage in a eukaryotic system was measured using both the Aspergillus methionine system and a diploid bioassay. All three fractions induced a doubling of revertant colonies both with and without metabolic activation (Figures 26, 27, and 28). The frequency of induced mutations per survivor for the acid and base fractions at the highest exposure level with metabolic activation was 100 and three times, respectively, the response obtained in the absence of metabolic activation (Tables 25 and 26). For the neutral fraction at the highest dose level, the induced mutation frequencies were almost the same with or without metabolic activation (Table 27). The results in Figures 27 and 28 show that in the absence of metabolic activation, the surviving fraction and induced mutation frequencies for the base and neutral fractions are almost identical at all exposure levels. The maximum mutagenic response was obtained with the acid fraction at the greatest exposure time with metabolic activation (Figure 26). Thus, the acid, base, and neutral fractions of the PENT S waste induced a significant increase in point mutations at the methionine suppressor loci both with and without metabolic activation. While the addition of metabolic activitation to the assay system did increase the frequency of induced mutations, significant increases were also obtained in the absence of metabolic activation.

Additional tests were conducted on the fractions of the wood-preserving bottom sediment using A. nidulans diploid 20. The diploid assay, while more costly and time consuming than the prokaryotic assays, offers the advantage of detecting a range of genetic damage and genotoxic compounds comparable to that detected using in vitro mammalian cell culture bioassays (Kafer et al., 1982). The summary of the genotoxic effects observed in segregant colonies and induced by the fractions of the PENT S waste (Table 28) indicates that each waste fraction preferentially acted on a different group of genes in the diploid Aspergillus. The genotoxic constituents present in the acid fraction induced predominantly fawn and dark green segregants, whereas the base and neutral fraction induced yellow, fawn, and chartreuse

TABLE 23. CAPACITY OF FRACTIONS OF WOOD-PRESERVING WASTE TO INDUCE INCREASED LETHAL DAMAGE IN DNA REPAIR DEFICIENT STRAINS OF B. SUBTILIS

Waste Fraction	Metabolic Activation	Growth Inhibition (mm)					
		Strain: 168 wt	recE4	recA8	hcr-9	fh2006-7	mc-1
Acid	+	13.9	17.6*	14.6	15.3	15.0	14.2
10 mg/pt	-	12.0	14.6*	11.7	12.9	13.4	12.9
Base	+	0	0	1.2	0	1.1	1.5
10 mg/pt	-	0.38	0.35	0.68	0	0.2	0
Neutral	+	0.43	0.37	0.20	0	1.0	0
10 mg/pt	-	2.3	2.2	3.0	1.3	2.3	2.1

* Significantly Inhibited.

TABLE 23. CAPACITY OF FRACTIONS OF WOOD-PRESERVING WASTE TO INDUCE INCREASED LETHAL DAMAGE IN DNA REPAIR DEFICIENT STRAINS OF B. SUBTILIS

Waste Fraction	Metabolic Activation	Growth Inhibition (mm)					
		Strain: 168 wt	recE4	recA8	hcr-9	fh2006-7	mc-1
Acid	+	13.9	17.6*	14.6	15.3	15.0	14.2
10 mg/pt	-	12.0	14.6*	11.7	12.9	13.4	12.9
Base	+	0	0	1.2	0	1.1	1.5
10 mg/pt	-	0.38	0.35	0.68	0	0.2	0
Neutral	+	0.43	0.37	0.20	0	1.0	0
10 mg/pt	-	2.3	2.2	3.0	1.3	2.3	2.1

* Significantly Inhibited.

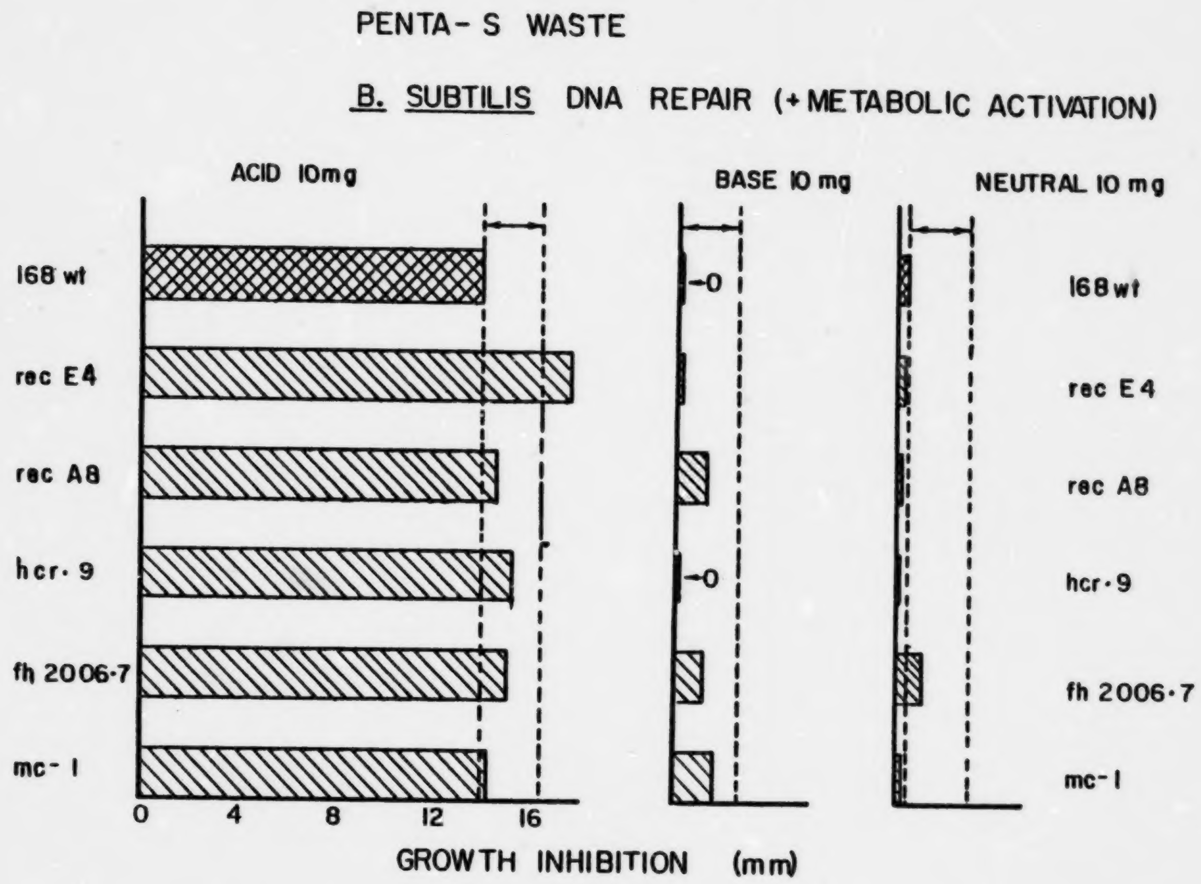


Figure 25. Growth inhibition induced by fractions of the wood-preserving bottom sediment in repair proficient (168 wt) and deficient strains of B. subtilis.

TABLE 24. FRACTIONAL SURVIVAL OF REPAIR PROFICIENT AND DEFICIENT STRAINS OF B. SUBTILIS EXPOSED TO SUBFRACTIONS OF WOOD-PRESERVING BOTTOM SEDIMENT. SURVIVAL WAS MEASURED IN THE PRESENCE OF METABOLIC ACTIVATION

Sample	Dose/Plate (µg)	Fractional Survival (N/N_o) %			
		168 wt RP	fh2006.7	recE4	mc-1
			repair deficient		
Acid	100	35 ± 28	19 ± 13	6 ± 4	NT*
	50	88 ± 28	34 ± 22	26 ± 28	NT
	10	92 ± 12	50 ± 17	41**	NT
Base	1000	20 ± 3	3 ± 2	2 ± 0.5	4 ± 1.5
	100	43 ± 28	13 ± 11	4 ± 0.5	33 ± 12.0
	10	>100	63 ± 15	9 ± 1.5	16 ± 2.0
Neutral	1000	5 ± 5	6 ± 5	2**	76**
	100	17 ± 6	15 ± 6	2**	64**
	10	66 ± 44	43 ± 12	36**	58**

* - NT = not tested

**- Standard deviation not provided because only one test conducted.

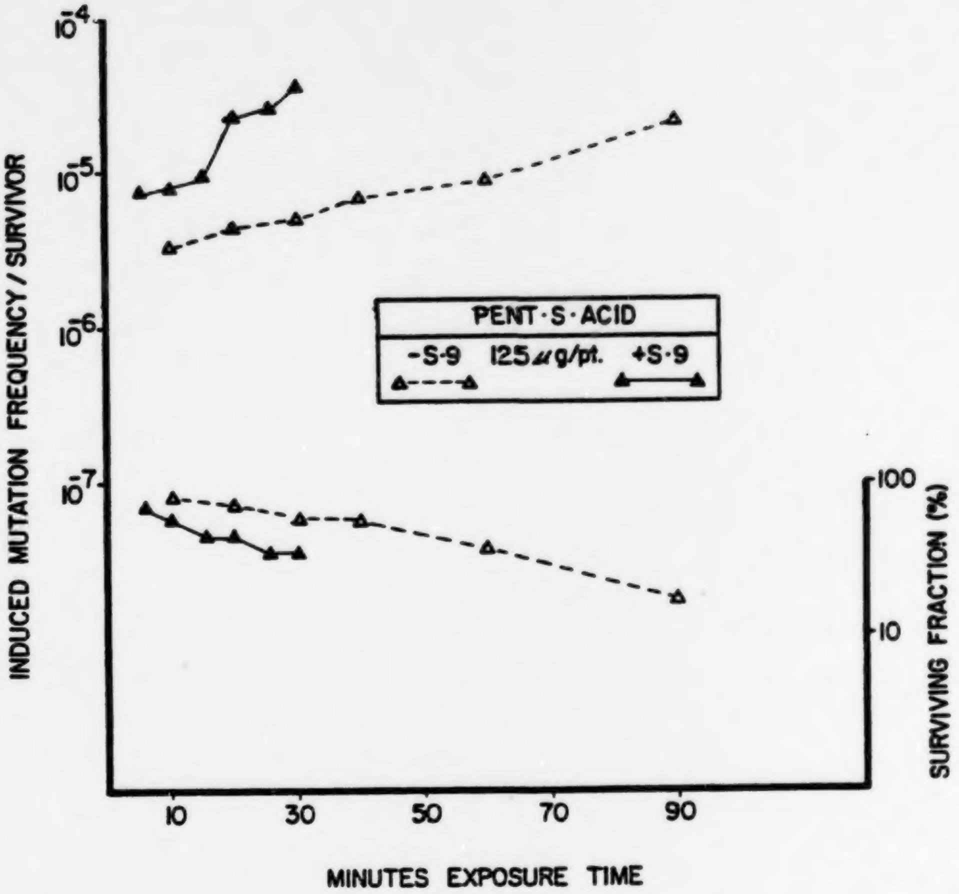


Figure 26. Induced mutation frequency and fractional survival in A. nidulans following exposure to acid fraction of PENT S waste. Data based on results from two independent experiments.

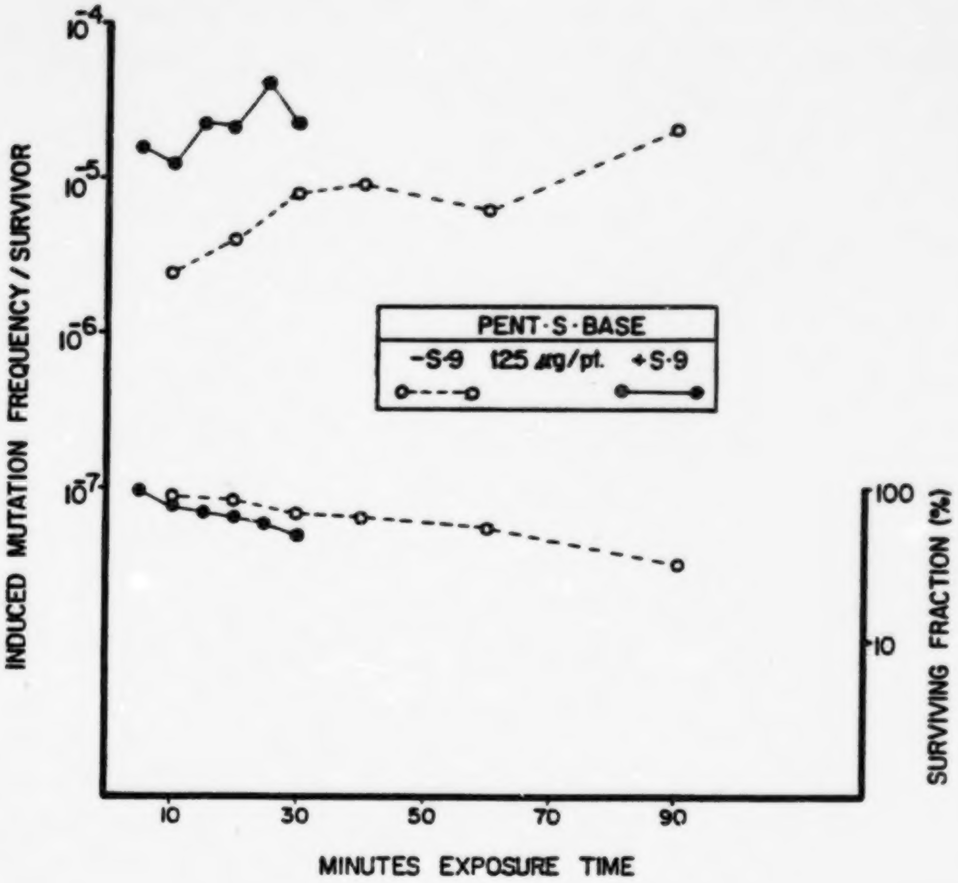


Figure 27. Induced mutation frequency and fractional survival in *A. nidulans* following exposure to base fraction of PENT S waste. Data based on results from two independent experiments.

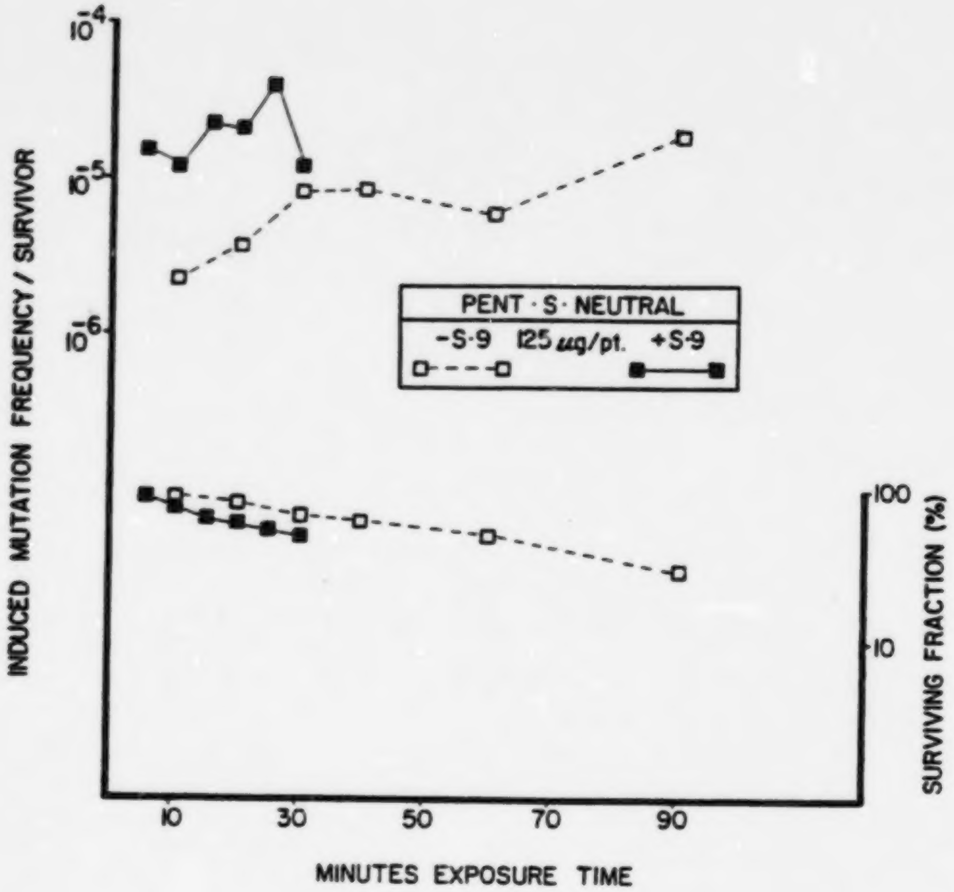


Figure 28. Induced mutation frequency and fractional survival in A. nidulans following exposure to neutral fraction of PENT S waste. Data based on results from two independent experiments.

TABLE 25. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF *A. NIDULANS* FOLLOWING EXPOSURE TO ACID FRACTION OF PENT S WASTE

Dose/Plate (µg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
41.7	40	.75	.84	1.4	.51	2.7
62.5	40	.75	1.4	1.8	.40	3.6
125.0	40	.54	2.4	3.6	.90	6.9
250.0	40	.31	5.4	6.8	2.5	14.9
With Metabolic Activation						
41.7	20	.75	1.9	3.2	.51	5.5
62.5	20	.82	2.2	4.2	.93	7.3
125.0	20	.56	3.8	8.7	1.3	13.9
250.0	20	.26	7.1	17.6	1.4	26.3

TABLE 26. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF *A. NIDULANS* FOLLOWING EXPOSURE TO BASIC FRACTION OF PENT S WASTE

Dose/Plate (µg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
41.7	40	.76	.98	1.7	.50	3.2
62.5	40	.94	1.6	6.1	.88	8.6
125.0	40	.64	3.1	4.2	.98	8.3
250.0	40	.40	2.4	6.9	1.4	10.7
With Metabolic Activation						
41.7	20	.82	1.6	2.7	.46	4.9
62.5	20	.91	2.8	9.5	1.3	13.6
125.0	20	.64	6.7	10.6	3.2	20.6
250.0	20	.40	10.4	17.6	3.9	31.9

TABLE 27. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF *A. NIDULANS* FOLLOWING EXPOSURE TO NEUTRAL FRACTION OF PENT S WASTE

Dose/Plate (µg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
41.7	40	.77	.63	1.8	.16	2.6
62.5	40	.83	1.3	5.3	.17	6.8
125.0	40	.57	.96	3.4	.22	4.6
250.0	40	.44	4.1	12.8	.50	17.4
With Metabolic Activation						
41.7	20	.70	3.3	6.0	.21	9.5
62.5	20	.78	.74	3.1	.37	4.2
125.0	20	.40	3.5	9.3	.91	13.7
250.0	20	.34	5.4	12.4	1.1	18.9

TABLE 28. SUMMARY OF GENOTOXIC EFFECTS OBSERVED IN SEGREGANT COLONIES OF DIPLOID A. NIDULANS FOLLOWING EXPOSURE TO WASTE CONTROLS AND FRACTIONS

			Phenotype[2,3]											ISI[4]	
			Yellow			Fawn		CHAT		DG		GR		CT	DO
Sample	Total[1]	Overall	CO	ND	M	2n	n	2n	n	2n	n	2n	n		
Control	619	1.9	0.3	0.5	0	0.3	0	0.3	0	0.3	0	0.2	0	_	.04
DMSO	140	4.0	0.7	0.7	0	0	0.7	2.0	0	0	0	0	0	1.4	-
Benomyl	299	12.7	1	3.5	0.3	1.4	0.3	2.3	0.7	1.0	1.3	1.0	0	5.2	3.7
Pent S - Acid	248	4.4	0	0	0	0.8	0.8	0.4	0	0.8	0.8	0.4	0.4	4.2	2.9
Base	306	10.0	2	1.0	0	1.6	1.3	2.6	0.3	0	0.3	1.0	0	1.6	1.1
Neutral	318	6.9	0.6	1.9	0	1.3	0.3	1.9	0	0	0	0.9	0	2.6	1.8
SWRI - Acid	369	11.1	1.6	2.7	0	1.4	0	2.4	0	0.3	1.4	1.4	0	2.7	1.9
Base	348	10.9	1.4	1.0	0	2	0.6	2.0	0	0.9	1.4	0.6	0.3	2.1	1.5
Neutral	332	9.6	2.1	2.7	0	1.5	0.3	2.4	0	0	0	0.6	0.0	2.6	1.8
COMBO - Acid	285	14.7	3.9	3.2	0	2.1	0.4	1.4	0	0	2.5	1.4	0	1.6	1.1
Base	285	11.2	1.8	3.5	0	2.5	0	1.1	0	0	1.8	0.7	0	1.9	1.3
Neutral	261	11.5	1.1	2.7	0	3.1	1.5	1.1	0.4	0	0.4	0.8	0.4	2.3	1.6

1. Total = number of segregant colonies examined; overall = total number of induced segregants.
2. Unit = Total frequency of segregants per colony examined.
3. Phenotype: Yellow-includes only colonies that were completely yellow, all other listed in other sections; CO = crossing-over, ND =non-disjunction, M = mutations, Fawn = tan sectors; Chat = chatreuse; DG = dark green G = green; 2n = diploid; n = haploid.
4. Induced segregation index = Total induced segregants divided by frequency of control segregants. CT = evaluated with respect to controls; DO = evaluated with respect to solvent control (DMSO). If ISI is significantly greater than 1.0, response is considered positive.

segregants (Table 28). Both crossing over and non-disjunction were observed in the yellow segregants induced by the base fraction, while predominantly non-disjunction was observed in yellow segregants from the neutral fraction. No yellow segregants were induced by the acid fraction. When the induced segregation indexes are compared with respect to the spontaneous control, all three waste fractions are positive with the genotoxic potential of the acid fraction slightly less than benomyl, the positive control. When the responses are compared with respect to the solvent control, the acid and neutral fractions are positive; while the induced segregation index of the base fraction, which is slightly greater than one, would be considered a borderline positive response.

In abnormal colonies, the acid, base, and neutral fractions induced 31, 15 and 25 abnormal colonies, respectively (Table 29). For all three fractions, the maximum mutation frequency was observed in the hyperdiploid class. The acid fraction induced significant increases in hyperdiploid colonies, breakdown of the mitotic spindle apparatus, and mitotic recombination; the neutral fraction induced significant increases in the hyperdiploid class and mitotic recombination. Thus, the acid fraction induced the maximum amount of genetic damage observed in abnormal colonies, while the neutral fraction also induced a significant positive response, and the base fraction induced a borderline positive response.

The results from biological analysis of the wood-preserving waste indicate that the base fraction induced the maximum response in both prokaryotic point mutation assays, the DNA repair plate incorporation assay, and the eukaryotic point mutation assay with metabolic activation. In the prokaryotic DNA repair spot test and the eukaryotic point assay, the acid fraction induced the maximum genotoxic response. Although the genotoxicity of the acid fraction was greatest in the presence of metabolic activation, a positive response was obtained in the absence of metabolic activation in both bioassays. The acid fraction, however, was toxic at higher dose levels in the *Salmonella* and *Bacillus* assays, possibly a result of the presence of pentachlorophenol. The acid fraction induced the maximum response in *S. typhimurium* strain 102. The wood-preserving bottom sediment contained a diverse range of constituents including compounds capable of causing induction, promotion, and inhibition, as well as mutagenic and carcinogenic effects (Figure 29, 30, 31, and Table 30). Biological analysis of the fractions of this waste detect compounds capable of causing point mutations, lethal damage to DNA, and various types of chromosome damage. While the results of biological testing do not rule out the PENT S waste as candidate for land treatment, the presence of pentachlorophenol and other compounds that are resistant to degradation or mobile in the soil indicate that land treatment should probably be conducted at a lower loading rate or be precluded by a pretreatment method such as composting or anaerobic digestion.

TABLE 29. SUMMARY OF GENOTOXIC EFFECTS OBSERVED IN ABNORMAL COLONIES OF DIPLOID *A. NIDULANS* FOLLOWING EXPOSURE TO CONTROLS AND WASTE FRACTIONS

		Genetic Damage Observed[1]					
Sample	Total[2]	OV	HD	NH	HH	BK	OT
CONTROL	COL=619	22	14	3	2	1	2
	MF	.036	.023	.005	.003	.002	.003
	ISI(S)	-	-	-	-	-	-
	ISI(D)	-	-	-	-	-	-
DMSO	COL=140	6	3	2	0	0	1
	MF	.043	.021	.014	-	-	.007
	ISI(S)	1.19	.91	2.8	-	-	2.3
	ISI(D)	-	-	-	-	-	-
BENOMYL	COL=299	47	19	3	1	19	5
	MF	.157	.050	.010	.003	.064	.017
	ISI(S)	4.36	2.17	2.0	1.0	32	5.67
	ISI(D)	3.65	2.38	.71	-	-	2.43
PENT S (Acid)	COL=248	31	21	3	0	2	5
	MF	.125	.085	.012	-	.008	.020
	ISI(S)	3.47	3.70	2.4	-	4.0	6.67
	ISI(D)	2.91	4.05	0.86	-	-	2.87
PENT S (Base)	COL=306	15	10	1	1	1	2
	MF	.049	.033	.003	.003	.003	.006
	ISI(S)	1.36	1.43	.06	1	1.5	2.0
	ISI(D)	1.14	1.57	.21	-	-	0.0

TABLE 29 CONTINUED

		Genetic Damage Observed[1]					
Sample	Total[2]	OV	HD	NH	HH	BK	OT
PENT S	COL=318	25	15	5	0	1	5
(Neutral)	MF	.079	.047	.016	-	.003	.013
	ISI(S)	2.19	2.04	3.2	-	1.5	4.3
	ISI(D)	1.84	2.24	1.14	-	-	1.86
SWRI	COL=369	28	16	4	1	2	5
(Acid)	MF	.076	.043	.011	.003	.006	.014
	ISI(S)	2.11	1.87	2.2	1	3	4.67
	ISI(D)	1.77	2.05	.78	-	-	2.00
SWRI	COL=348	27	19	2	0	0	6
(Base)	MF	.078	.055	.006	-	-	.017
	ISI(S)	2.17	2.39	1.2	-	-	5.67
	ISI(D)	1.81	2.05	.36	-	-	2.43
SWRI	COL=332	21	10	7	1	2	5
(Neutral)	MF	.063	.030	.021	.003	.006	.003
	ISI(S)	1.75	1.30	4.2	1	3	1
	ISI(D)	1.47	1.43	1.5	-	-	.42
COMBO	COL=285	14	7	2	0	4	1
(Acid)	MF	.049	.056	.007	-	.014	.004
	ISI(S)	1.36	1.09	1.4	-	7	1.3
	ISI(D)	1.14	1.19	.5	-	-	1.3

TABLE 29 CONTINUED

Sample	Total[2]	Genetic Damage Observed[1]					
		OV	HD	NH	HH	BK	OT
COMBO (Base)	COL=285	20	16	1	0	0	3
	MF	.70	.056	.004	-	-	.011
	ISI(S)	1.94	2.43	.8	-	-	3.66
	ISI(D)	1.63	2.67	.29	-	-	1.57
COMBO (Neutral)	COL=261	15	3	0	0	1	11
	MF	.057	011	-	-	.004	.042
	ISI(S)	1.58	.48	-	-	2	14
	ISI(D)	1.33	.52		-	-	6

1. Genetic damage observed: OV = overall summary of genetic damage observed in all categories; HD = hyperdiploid, results from non-disjunction of chromosomes; NH = near hyperdiploid, probably breakdown products including a mixture of haploid and diploid colonies; HH = hyperhaploid, results from major deletions or lethals in chromosomes; BK = breakdown, results from mitotic spindle poisons; OT = others, results from mitotic recombination which has occurred prior to aneuploidy. Units = Total frequency of abnormal segregants per colony examined.

2. Total: col = total number of colonies examined, and total number of abnormals in each class; MF = total mutation frequency = OV COL; ISI(S) = induced segregation index with respect to control = MF (sample) MF (control); ISI(D) = induced segregation index with respect to solvent control = MF(sample) MF(DMSO).

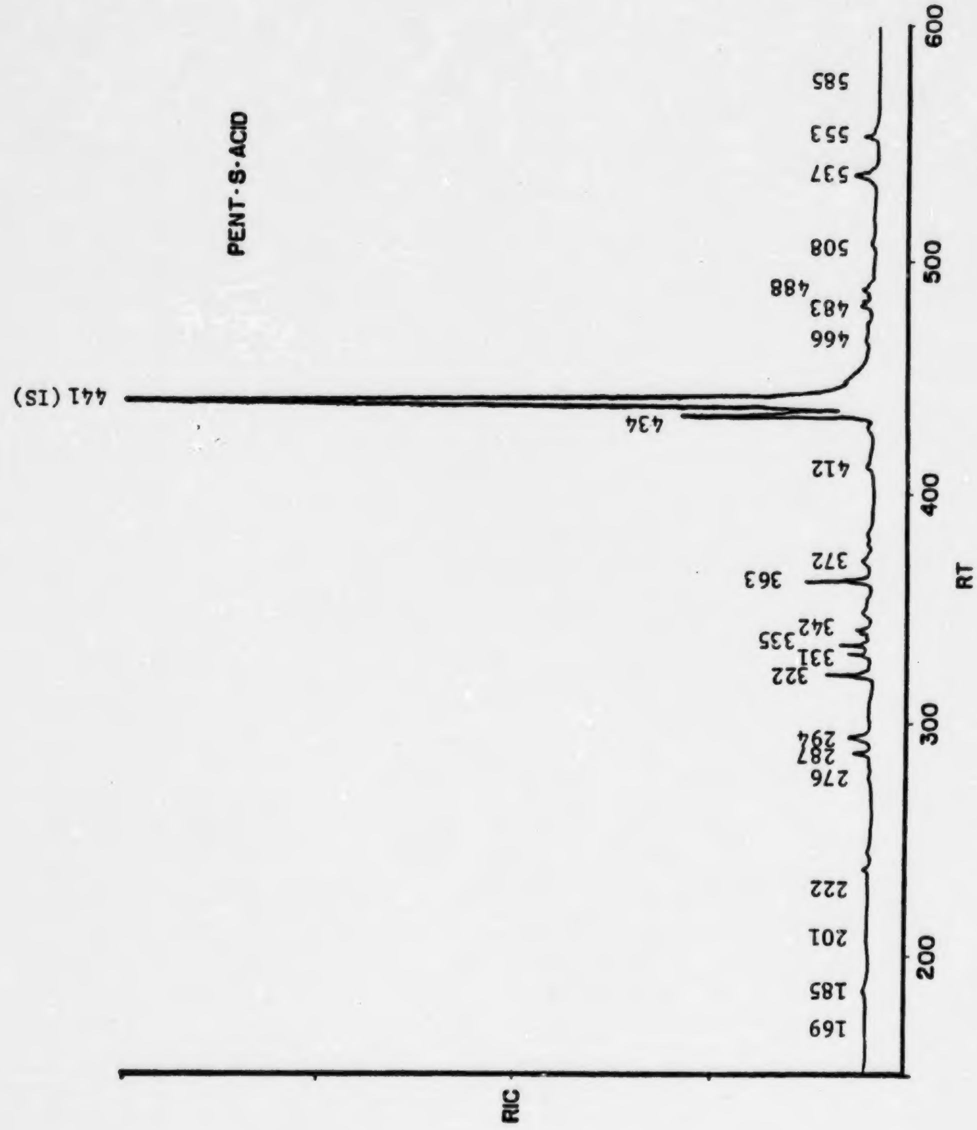


Figure 29. GC/MS chromatograph of acid fraction of PENT S waste.

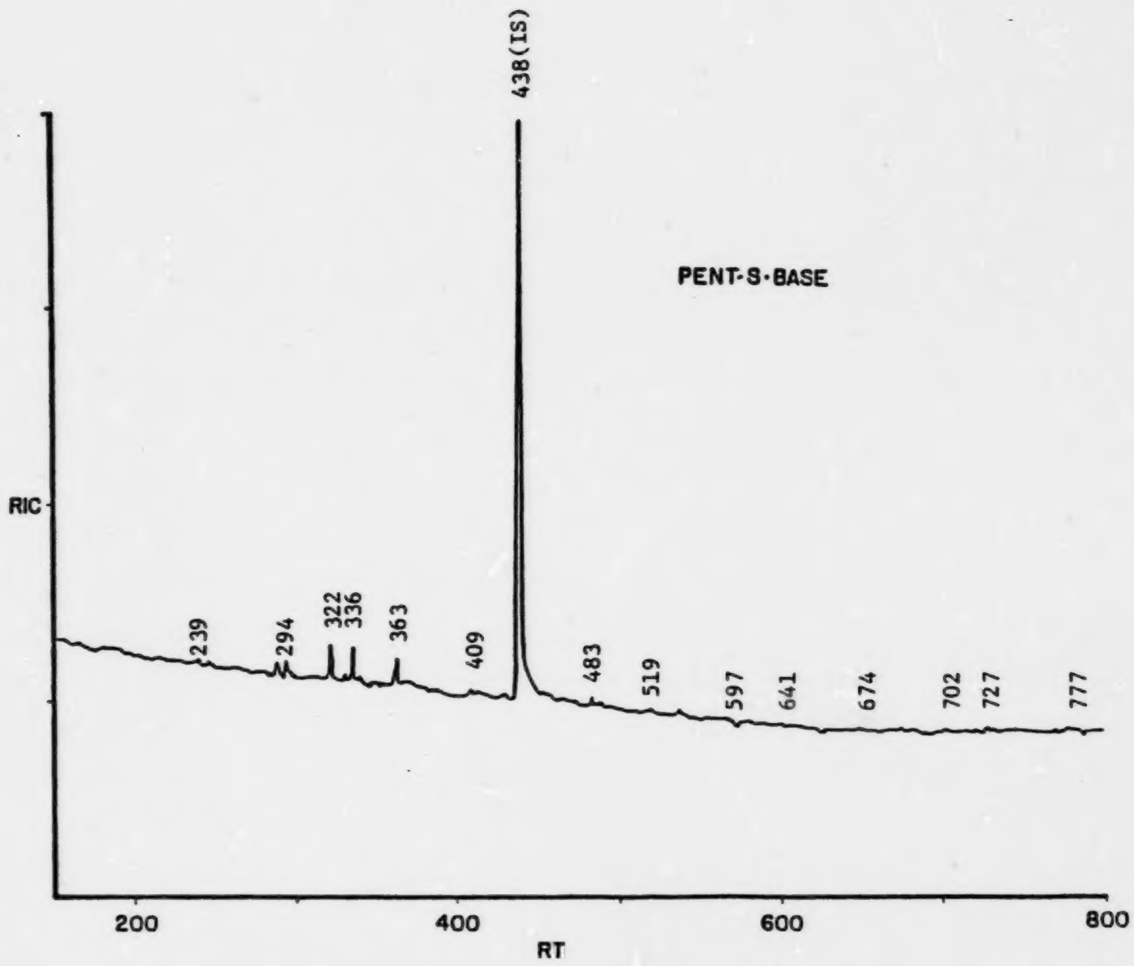


Figure 30. GC/MS chromatograph of base fraction of PENT S waste.

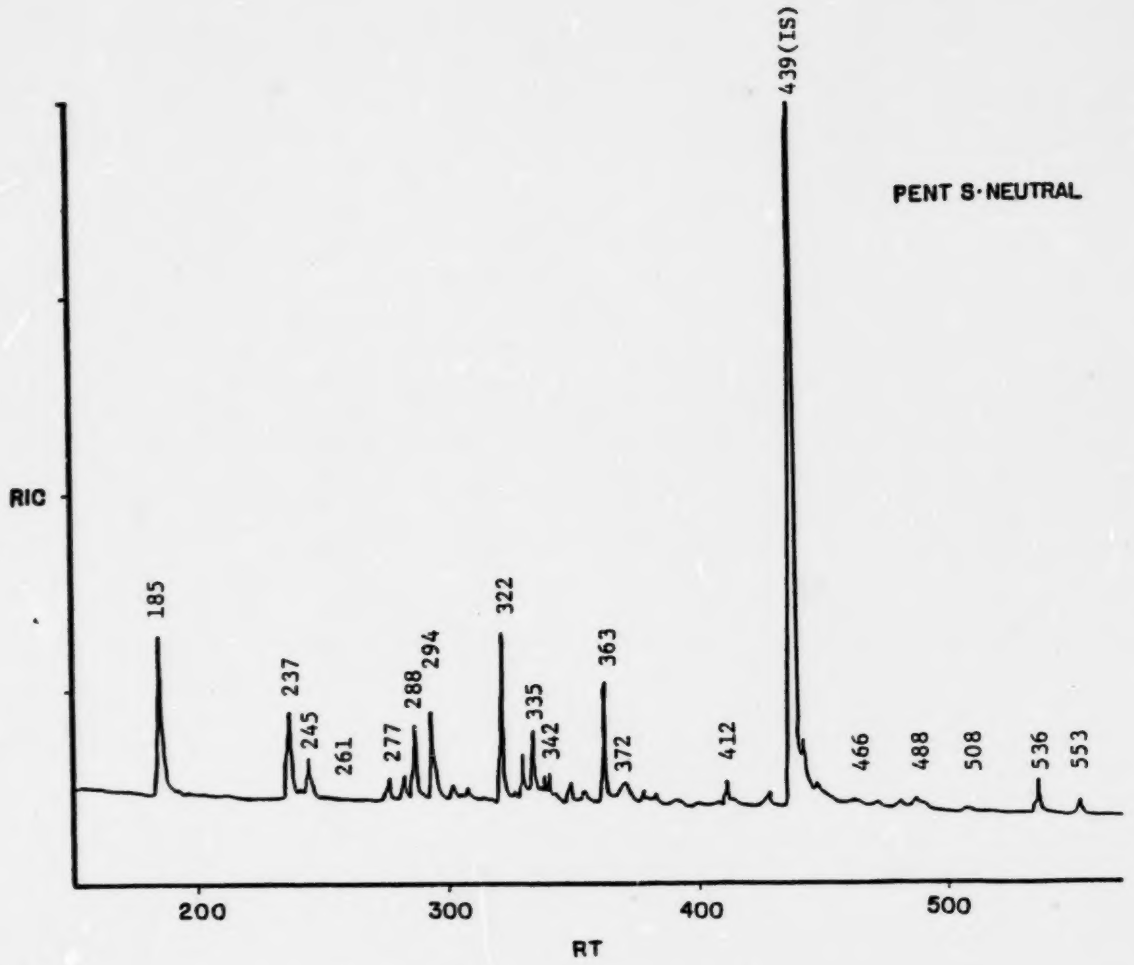


Figure 31. GC/MS chromatograph of neutral fraction of PENT S waste.

TABLE 30. SELECTED PROPERTIES OF COMPOUNDS IDENTIFIED IN THE FRACTIONS OF THE WOOD-PRESERVING WASTE

Sample	Peak Number	Compound	CAS #	Formula	Genetic Activity
Acid Fraction	287	Dimethyl napthalene	573-98-8	$C_{12}H_{12}$	MO
	322	1-2 Dihydro-acenapthylene	83-32-9	$C_{12}H_{10}$	Ml
	331	Methylethyl napthalene		$C_{13}H_{14}$	X
	335	Dibenzofuran	132-64-9	$C_{12}H_8O$	X
	342	Trimethyl napthalene	879-129	$C_{13}H_{14}$	MO
	363	Phenalene	203-80-5	$C_{13}H_{10}$	MO; Cl
	434	Pentachlorophenol	87-86-5	C_6HCL_5O	Ml
	483	Methyl phenanthrene	832-69-9	$C_{15}H_{12}$	Ml;CO;CC
	537	Fluoranthene	206-44-0	$C_{16}H_{10}$	Ml;CO;CC
	553	Pyrene	121-00-0	$C_{16}H_{10}$	Ml;CO
Basic Fraction	239	Methyl napthalene	90-12-0	$C_{11}H_{10}$	Ml;CO
	322	1-2 Dihydro-acenapthylene	83-32-9	$C_{12}H_{10}$	Ml
	336	Dibenzofuran	132-64-9	$C_{12}H_8O$	X
	363	Phenalene	203-80-5	$C_{13}H_{10}$	X
Neutral Fraction	185	Napthalene	91-20-3	$C_{10}H_8$	MO;CO
	237	Methyl napthalene	90-12-0	$C_{11}H_{10}$	Ml
	288	Dimethyl napthalene	573-98-8	$C_{12}H_{12}$	MO
	322	1-2 Dihydro-acenapthylene	83-32-9	$C_{12}H_{10}$	Ml
	335	Dibenzofuran	132-64-9	$C_{12}H_8O$	X
	342	Trimethyl napthalene	879-129	$C_{13}H_{14}$	MO
	363	Phenalene	203-80-5	$C_{13}H_{10}$	X
	536	Fluoranthene	206-44-0	$C_{16}H_{10}$	Ml,CO;CC
	553	Pyrene	121-00-0	$C_{16}H_{10}$	Ml;CO;CC

1. Potential genetic activity: P = promotor; I = inhibitor; CC = cocarcinogenic; Ml = mutagenic; MO = nonmutagenic; Cl = carcinogenic; CO = noncarcinogenic; X = unknown; references included in text.

The storm-water runoff impoundment (SWRI) waste was one of two refinery sludges evaluated in the complete battery of bioassays. The distribution of mutagenic activity in the fractions of the SWRI waste are provided in Table 31 and Figure 32. These results indicate that although the maximum response was obtained from the acid fraction, the specific activity of all three fractions was approximately equal. In addition, when the neutral fraction was separated on a silica gel column into subfractions, the specific activity of neutral fraction three was approximately three times the specific activity of the unfractionated neutral fraction (Figure 33). The dose-response curves for the extractable hydrocarbons of the SWRI waste fractions (Figure 32) also indicate that the overall maximum response was obtained from the acid fraction, although the maximum mutagenic response at a single dose level was obtained with the neutral fraction at a dose of 1.0 mg/plate. With the exception of the neutral fraction at the highest dose, there was no mutagenic activity detected in the absence of metabolic activation. In the three subfractions of the neutral fraction (Figure 33), higher levels of mutagenic activity were detected at the lower doses than were present in the composite neutral fraction. The maximum amount of mutagenic activity was detected in fraction three which represents the condensed ring fraction. The results from biological analysis of the SWRI waste indicate that predominantly indirect acting mutagens were detected in the waste fractions, and there was no appreciable difference in the mutagenic potential of each of the three primary fractions. The analysis of fractions of the storm-water runoff impoundment waste in the spot test or plate incorporation assay using DNA repair proficient and deficient strains of B. subtilis did not provide any conclusive results (Table 32). Thus, the storm-water runoff impoundment waste contained constituent(s) that were capable of inducing point mutations but not lethal damage to DNA.

The fractions of the SWRI waste were also evaluated in the Aspergillus methionine and diploid bioassays. When the results from testing the three waste fractions in the haploid methionine assay are compared, the maximum mutagenic response was obtained from the acid fraction in the presence of metabolic activation (Tables 33, 34 and 35; and Figures 34, 35, and 36). While a positive response was also obtained from the base and neutral fractions, the induced mutation frequencies were consistently lower than were obtained with the acid fraction. In contrast to the results of the prokaryotic bioassay, mutagenic responses were observed for all waste fractions in Aspergillus in the absence of metabolic activation.

Each of the three subfractions of the storm-water runoff impoundment waste induced a positive response in the diploid bioassay. The results presented in Table 28 indicate that in segregant colonies the maximum genotoxic response was obtained with the acid fraction, although the genotoxic potential of the neutral fraction was not significantly lower than the acid fraction. Yellow and chartreuse segregants were predominantly induced by the acid and neutral fraction,

TABLE 31. MUTAGENIC ACTIVITY OF FRACTIONS OF STORM-WATER RUNOFF IMPOUNDMENT

Fraction	Dose/plate (mg)	TA 98		TA 100		TA1535		TA1538		TA1537	
		S9 −	+	−	+	−	+	−	+	−	+
		Total his^+ revertants (Mean ± SD)									
Acid	0	26 ± 2	29 ± 6	115 ± 13	109 ± 7	28 ± 8	21 ± 4	12 ± 2	21 ± 4	9 ± 2	14 ± 2
	1	53 ± 18	222 ± 23	114 ± 19	249 ± 27	36 ± 10	28 ± 5	67 ± 8	75 ± 11	42 ± 4	42 ± 14
	.5	32 ± 5	158 ± 40	116 ± 11	255 ± 36	47 ± 16	38 ± 3	52 ± 14	78 ± 8	38 ± 5	44 ± 8
	.1	28 ± 7	88 ± 23	114 ± 25	245 ± 31	53 ± 15	25 ± 5	18 ± 5	61 ± 9	10 ± 2	33 ± 11
	.05	24 ± 7	68 ± 8	102 ± 11	198 ± 10	49 ± 27	25 ± 4	19 ± 7	42 ± 17	15 ± 4	26 ± 14
	.01	23 ± 6	34 ± 10	105 ± 24	133 ± 12	36 ± 17	24 ± 5	14 ± 2	17 ± 3	13 ± 3	25 ± 3
Base	0	26 ± 2	45 ± 7	130 ± 5	124 ± 6	28 ± 8	20 ± 4	12 ± 2	25 ± 2	10 ± 2	24 ± 5
	1	47 ± 18	151 ± 30	126 ± 9	349 ± 49	52 ± 17	28 ± 2	34 ± 6	111 ± 51	25 ± 6	27 ± 8
	.5	34 ± 3	144 ± 23	137 ± 7	337 ± 120	52 ± 16	28 ± 4	23 ± 6	90 ± 30	16 ± 5	22 ± 8
	.1	31 ± 6	82 ± 12	109 ± 11	230 ± 43	41 ± 12	20 ± 5	17 ± 5	46 ± 14	13 ± 2	20 ± 5
	.05	28 ± 6	68 ± 9	112 ± 17	185 ± 12	38 ± 12	22 ± 5	16 ± 3	25 ± 18	11 ± 5	21 ± 6
	.01	26 ± 3	44 ± 9	113 ± 10	132 ± 21	37 ± 6	21 ± 5	18 ± 5	19 ± 5	16 ± 2	13 ± 1
Neutral	0	25 ± 2	44 ± 6	125 ± 9	109 ± 7	28 ± 8	19 ± 2	12 ± 2	25 ± 4	10 ± 2	21 ± 6
	1	79 ± 44	255 ± 111	147 ± 11	290 ± 26	21 ± 6	25 ± 7	26 ± 5	66 ± 11	20 ± 12	42 ± 10
	.5	49 ± 21	134 ± 25	123 ± 16	283 ± 14	23 ± 7	22 ± 4	20 ± 3	52 ± 2	14 ± 3	43 ± 17
	.1	28 ± 13	74 ± 10	115 ± 9	227 ± 21	26 ± 1	24 ± 3	16 ± 1	20 ± 5	12 ± 4	39 ± 16
	.05	25 ± 9	66 ± 24	120 ± 6	166 ± 54	20 ± 6	19 ± 4	14 ± 2	32 ± 7	10 ± 3	28 ± 10
	.01	36 ± 10	50 ± 7	130 ± 15	119 ± 19	21 ± 7	23 ± 6	14 ± 2	17 ± 3	8 ± 3	25 ± 7

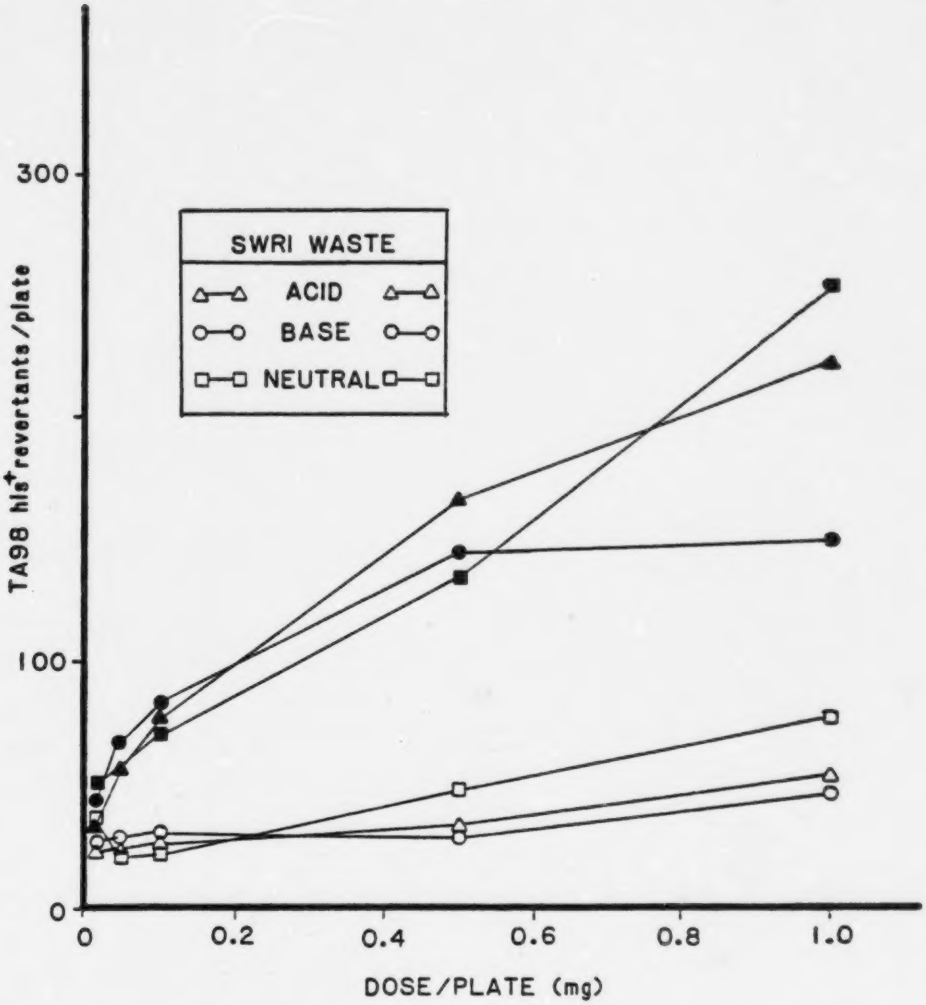


Figure 32. Mutagenic activity of fractions of SWRI waste.

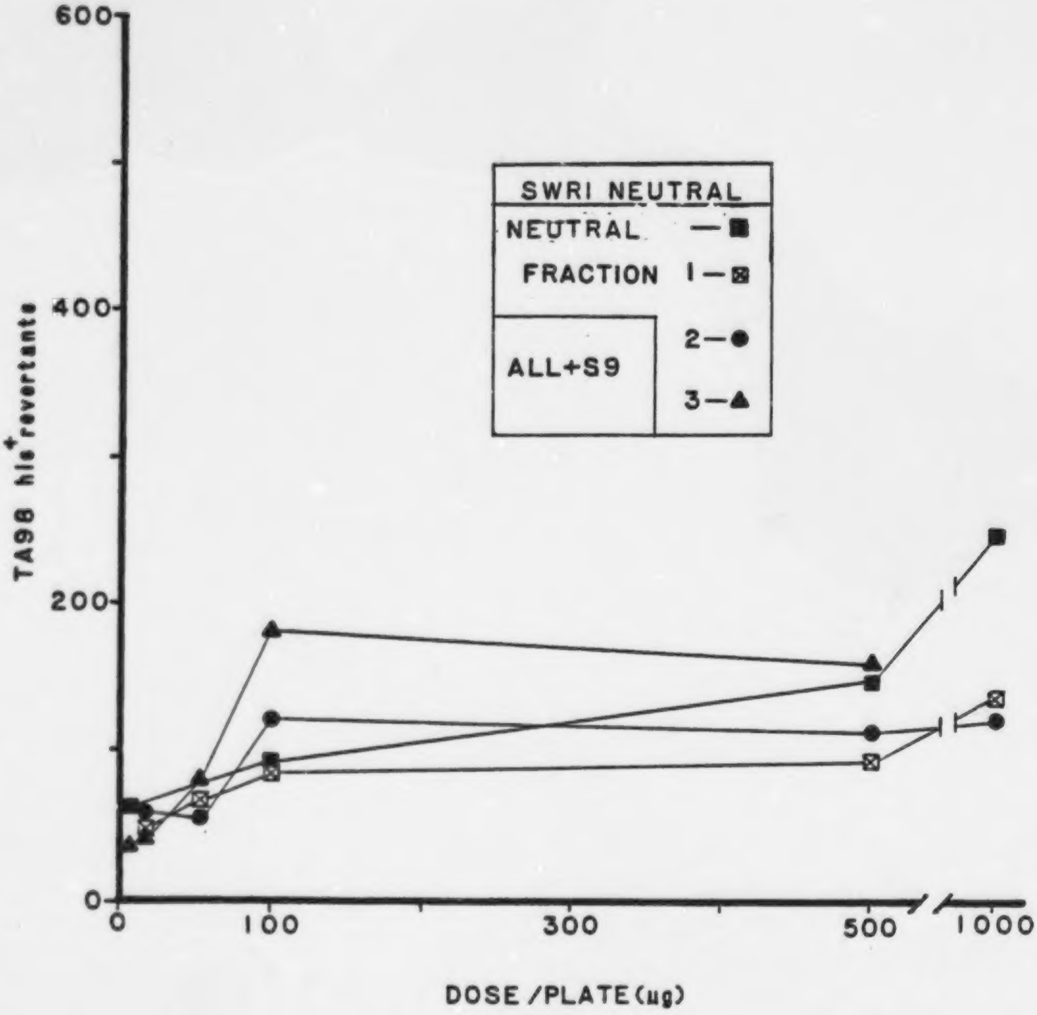


Figure 33. Mutagenic activity of subfractions of neutral fraction of SWRI waste.

TABLE 32. FRACTIONAL SURVIVAL OF REPAIR PROFICIENT (168 WT) AND DEFICIENT STRAINS OF _B. SUBTILIS_ EXPOSED TO SUB-FRACTIONS OF COMBINED API-SEPARATOR/SLOP-OIL EMULSION SOLID (COMBO) OR STORM-WATER RUNOFF IMPOUNDMENT (SWRI). SURVIVAL WAS MEASURED IN THE PRESENCE OF METABOLIC ACTIVATION

Sample	Dose/Plate (µg)	Fractional Survival (N/N_o) %		
		168 wt RP	hcr-9	recA8
			-- repair deficient -	
COMBO				
Acid	500	72 ± 0.6	27 ± 9	NT*
	100	97 ± 8	54 ± 5	
	50	>100 ± 24	55 ± 17	
	10	96 ± 4	>100**	
Base	1000	31**	27 ± 13	NT*
	500	43 ± 27	64 ± 10	
	100	87 ± 14	60 ± 8	
	50	>100	50 ± 21	
SWRI				
Acid	2500	8**	21**	32**
	1000	52 ± 33	53	66
	500	64 ± 14	NT	>100
	100	100 ± 0	>100 ± 0	>100
	50	96 ± 8	>100 ± 0	

* - NT = not tested

**- Standard deviation not provided because only one test conducted.

TABLE 33. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF A. NIDULANS FOLLOWING EXPOSURE TO ACID FRACTION OF SWRI WASTE

Dose/Plate (μg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
99	40	.54	7.7	7.2	1.0	16.0
199	40	.24	30.0	19.0	2.3	51.0
248	40	.23	30.7	20.3	3.9	54.9
497	40	.03	124.0	88.6	8.9	221.5
With Metabolic Activation						
124	20	.44	119.0	67.4	35.4	221.9
248	20	.17	44.4	37.2	10.5	92.1
331	20	.29	36.2	31.1	1.4	68.6
497	20	.09	87.1	61.0	8.7	156.9

TABLE 34. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF *A. NIDULANS* FOLLOWING EXPOSURE TO BASE FRACTION OF SWRI WASTE

Dose (μg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
42	40	.78	0	1.7	0	1.7
84	40	.46	1.9	0.7	0	2.6
168	40	.21	7.8	19	0	26.8
226	40	.12	9.9	26	0	35.8
336	40	.04	91.0	242	15	348
With Metabolic Activation						
42	20	.76	0	3.4	0	3.4
84	20	.61	7.0	1.3	0.8	9.1
128	20	.39	8.4	2.7	1.6	12.7
168	20	.23	24	14	5.8	44.0
226	20	.21	8.6	25	0	33.6

TABLE 35. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF A. NIDULANS FOLLOWING EXPOSURE TO NEUTRAL FRACTION OF SWRI WASTE

Dose (µg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
93	40	.94	1.2	1.1	0	2.3
185	40	.82	3.6	1.0	0	4.6
463	40	.51	6.2	1.3	0	7.5
719	40	.35	9.8	2.1	0	11.9
925	40	.41	13.0	2.7	0.5	16.2
With Metabolic Activation						
185	20	.74	9.5	2.4	0	11.9
370	20	.67	4.9	0	0.6	5.5
555	20	.49	13	1.9	1.3	16.2
719	20	.45	14	2.9	0	16.9
925	20	.40	11	1.4	1.2	13.6

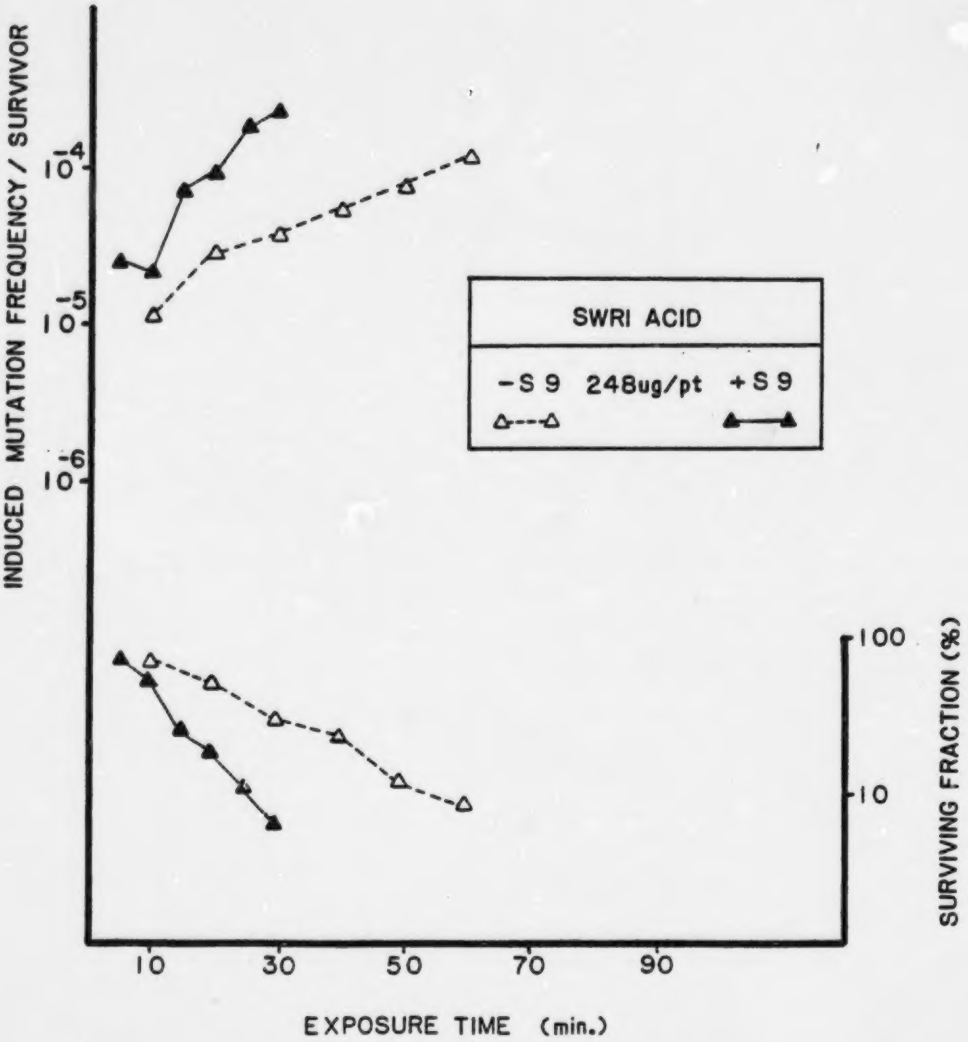


Figure 34. Induced mutation frequency and fractional survival in *A. nidulans* following exposure to acid fraction of SWRI waste. Data based on results from two independent experiments.

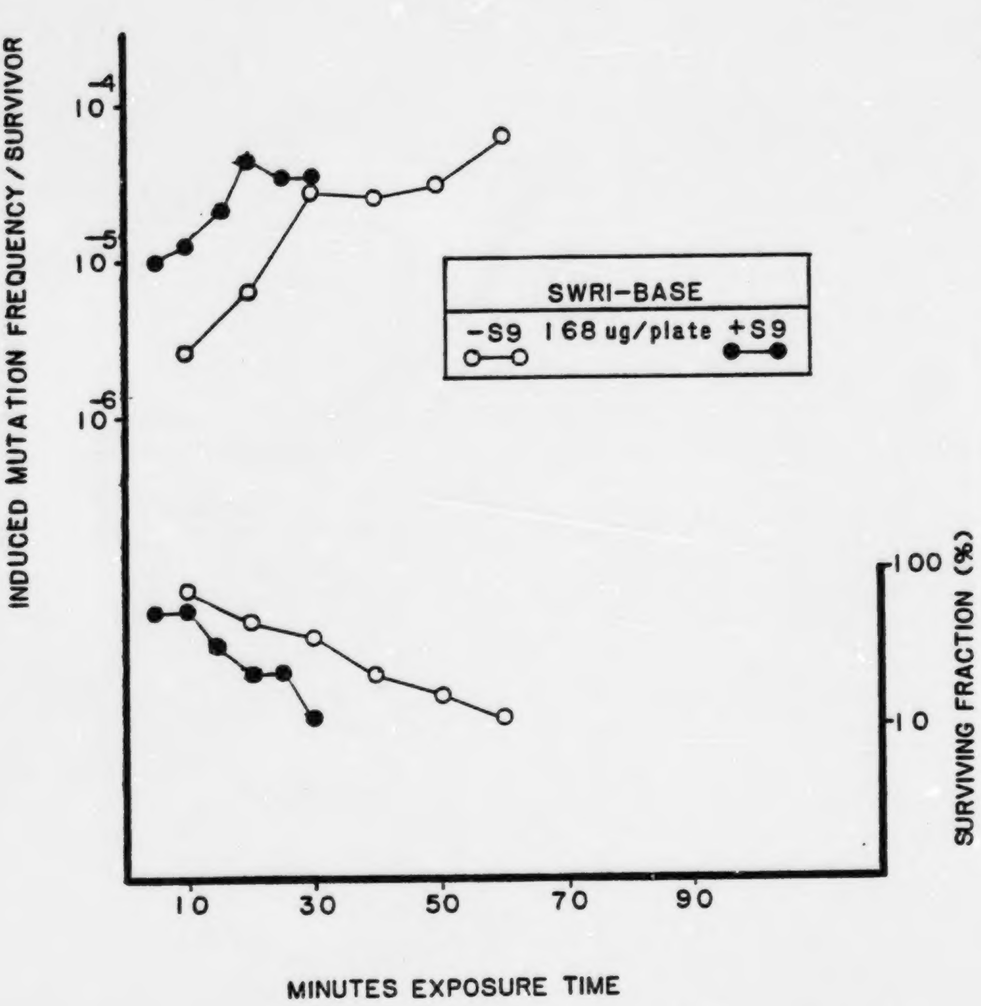


Figure 35. Induced mutation frequency and fractional survival in A. nidulans following exposure to base fraction of SWRI waste. Data based on results from two independent experiments.

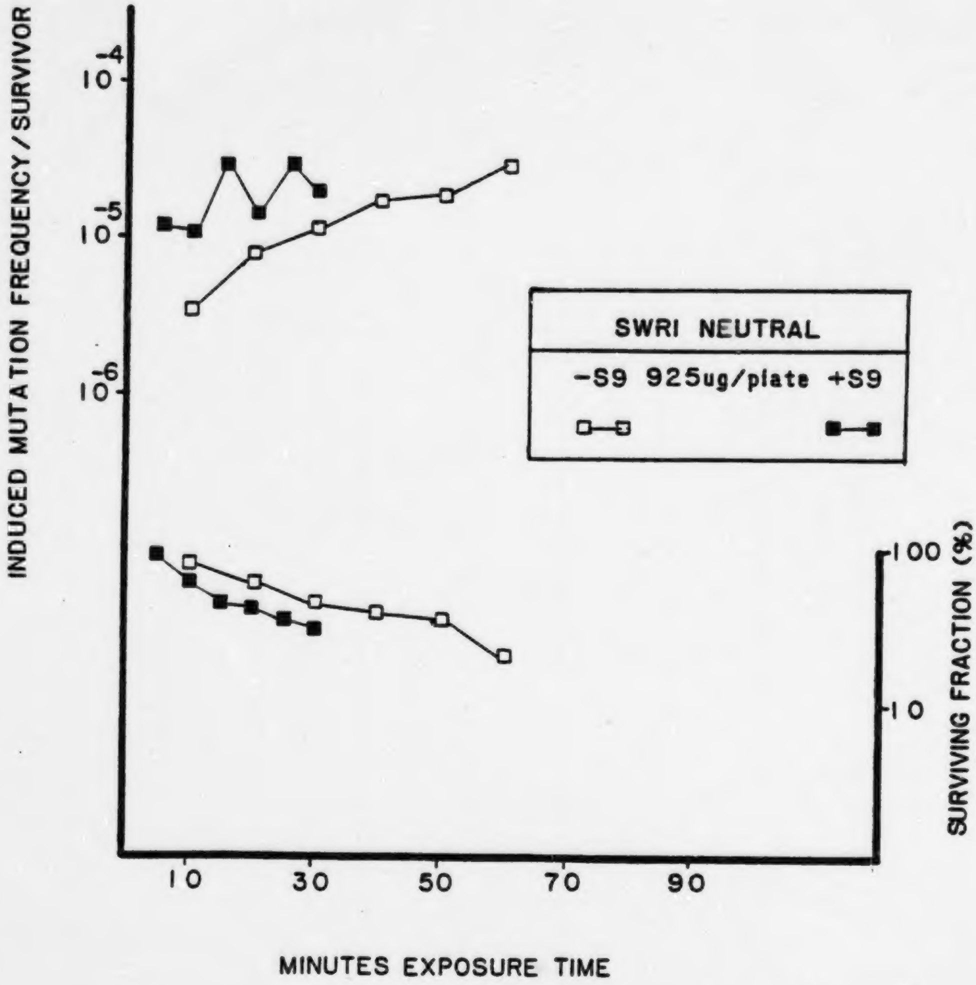


Figure 36. Induced mutation frequency and fractional survival in A. nidulans following exposure to neutral fraction of SWRI waste. Data based on results from two independent experiments.

while the base fraction induced a range of segregant colonies including all possible phenotypes. The majority of yellow segregants induced by the acid and neutral fractions were a result of non-disjunctional events, while yellow segregants induced by the base fraction resulted predominantly from crossing over. In addition, a relatively high frequency of diploid colonies were recovered from chartreuse segregants induced by each of the three fractions (Table 28). The results from the diploid assay demonstrate that constituent(s) of the fractions of the SWRI waste are capable of inducing a broad range of genotoxic damage in segregant colonies.

In abnormal colonies, the observed induced segregation index with respect to DMSO for the acid, base, and neutral fractions of the SWRI waste was 1.77, 1.81 and 1.47, respectively (Table 29). The acid and base fractions induced primarily hyperdiploid colonies and mitotic recombination, while the neutral fraction induced hyperdiploid and near hyperdiploid colonies. All three fractions of the SWRI waste induced a positive response in the abnormal colonies. In addition, the maximum genotoxic response was induced by the base fraction, although the induced segregation index of the acid fraction was not significantly lower.

The results from the chemical analysis of the fractions of the storm-water runoff impoundment waste were far less conclusive. The components of the acid, base, and neutral fraction of the SWRI waste were present at levels that were below the detection limits of the procedures used. Thus, the results from the chemical analysis of the waste fractions are, to a limited extent, in contrast to the results from biological testing.

Biological analysis detected genotoxic constituent(s) in all three fractions of the storm-water runoff impoundment waste, and three of the four bioassays detected the maximum genotoxic response in the acid fraction with metabolic activation. Genotoxic constituent(s) in the SWRI waste were capable of inducing point mutations and chromosome damage; however, the concentrations of the genotoxic compounds in the waste were below the detection limits of the analytical instruments. The potential of the SWRI waste to be treated by land application should be enhanced by the fact that genotoxic compounds are present in such low concentrations. Since adsorption and degradation are frequently concentration dependent, the SWRI waste can possibly be treated by land application, in spite of the presence of genotoxic constituents. However, because of the inability of chemical analysis to identify compounds present at genotoxic levels in the waste, short-term bioassays appear to provide the only analytical method capable of efficiently monitoring land application of this waste.

The second refinery waste evaluated in this research was a combined API separator slop-oil emulsion solids (COMBO) waste. The dose-response curves for the acid, base, and neutral fractions, as measured in the *Salmonella*/microsome assay are provided in Figure 37 and Table 36. These results indicate that the maximum mutagenic response was obtained from the neutral fraction in the presence of metabolic activation and that significant quantities of mutagenic activity were also detected in the neutral fraction in the absence of metabolic activation. In addition, none of the subfractions of the neutral fraction induced a greater mutagenic response than the whole fraction (Figure 38). Tests were also conducted with the base fraction using phenobarbitol induced rat liver. Epler *et al*. (1978) found that Aroclor-induced rat liver reacted best with the neutral fraction, while phenobarbitol-induced rat liver reacted best with the base fraction of synthetic fuel extracts. However, the results in Figure 39 indicate that for both strains TA98 and TA100, the maximum frequency of induced mutations was observed in the presence of Aroclor 1254 induced S9. Thus, all three waste fractions induced a mutagenic response in the *Salmonella* assay.

The analysis of fractions of the combined API-separator/slop oil emulsion waste in the spot test using DNA repair deficient and proficient bacteria did not provide any conclusive results (data not shown). The use of the modified plate incorporation assay (Donnelly *et al*., 1983) did, however, provide more conclusive results. The acid fraction induced the maximum response in the modified DNA repair assay (Table 32). The survival ratio of the repair proficient strain (168) compared to the repair deficient strain (hcr-9) was 0.37 for the acid fraction, while the base fraction induced a survival ratio of 0.69. There was no appreciable difference in the survival of the repair proficient and deficient strains following exposure to the neutral fraction.

Mutagenic activity was also detected in the fractions of the COMBO using the *Aspergillus* methionine assay (Tables 37, 38 and 39 and Figures 40, 41 and 42). The maximum mutagenic response was obtained from the neutral fraction with metabolic activation, and mutagenic activity was also detected in the absence of metabolic activation (Figure 42). The neutral fraction induced 2575 mutations/10^{-6} survivors. This was the maximum mutagenic response induced in the *Aspergillus* methionine system by any of the waste fractions tested in the present study. Mutagenic activity was also detected in the acid fraction both with and without metabolic activation; whereas, the base fraction induced a positive response only in the presence of metabolic activation (Figures 40 and 41). In the diploid system, the neutral fraction of the COMBO waste induced the maximum genotoxic response. Significant increases in the induced segregation index were also observed in the base fraction; whereas, the induced segregation index in the acid fraction, with respect to DMSO, would be considered a borderline positive response. The acid and base fractions induced predominantly yellow segregants, while constituent(s) of the neutral fraction appeared to preferentially induce fawn segregants (Table 28). The overall mutation frequencies in abnormal colonies induced by the

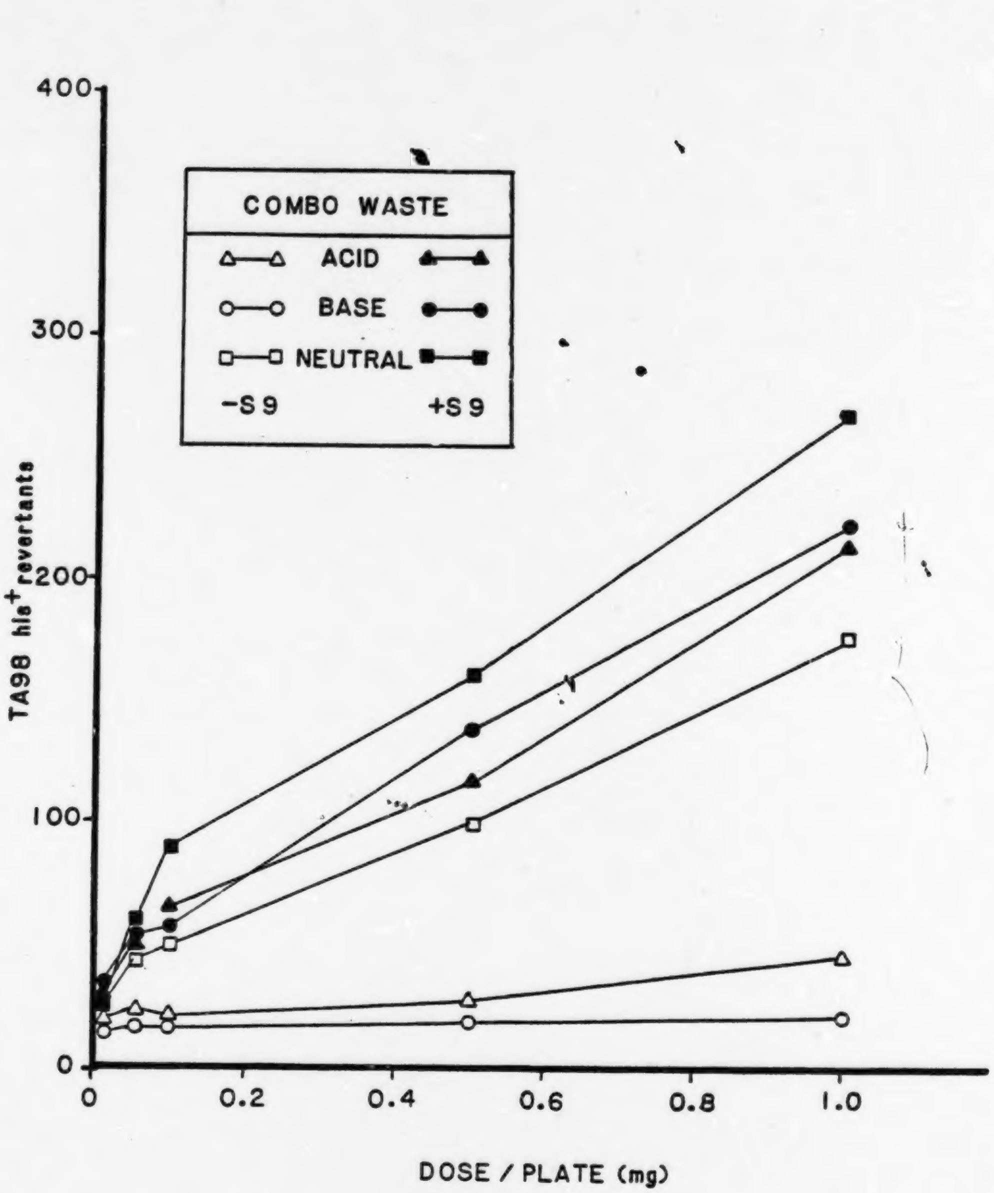


Figure 37. Mutagenic activity of fractions of COMBO waste.

TABLE 36. MUTAGENIC ACTIVITY OF FRACTIONS OF COMBINED API SEPARATOR/SLOP OIL EMULSION WASTE

Fraction	Dose/plate (mg)	TA 98		TA 100		TA1535		TA1538	
		S9 -	+	-	+	-	+		+
		Total his$^+$ revertants (Mean ± SD)							
Acid	0	24 ± 2	27 ± 6	99 ± 6	99 ± 16	22 ± 3	18 ± 1	13 ± 3	24 ± 4
	1	45 ± 26	217 ± 37	98 ± 16	118 ± 7	28 ± 6	24 ± 4	17 ± 2	20 ± 1
	.5	26 ± 12	118 ± 13	102 ± 9	128 ± 24	27 ± 10	29 ± 7	19 ± 3	49 ± 12
	.1	22 ± 9	65 ± 10	86 ± 16	144 ± 29	34 ± 13	29 ± 6	13 ± 3	25 ± 17
	.05	24 ± 4	51 ± 15	86 ± 19	121 ± 31	30 ± 9	20 ± 4	16 ± 4	32 ± 5
	.01	22 ± 3	31 ± 13	78 ± 10	103 ± 23	27 ± 12	20 ± 3	26 ± 17	26 ± 3
Base	0	17 ± 1	25 ± 7	85 ± 11	114 ± 13	23 ± 4	23 ± 4	13 ± 3	25 ± 4
	1	24 ± 6	224 ± 13	100 ± 41	NT*	22 ± 13	28 ± 3	14 ± 2	290 ± 86
	.5	21 ± 4	139 ± 70	108 ± 19	207 ± 35	34 ± 9	35 ± 8	17 ± 7	185 ± 56
	.1	20 ± 10	53 ± 10	85 ± 16	138 ± 29	39 ± 11	27 ± 6	13 ± 4	58 ± 8
	.05	22 ± 6	52 ± 3	95 ± 9	112 ± 19	39 ± 9	18 ± 5	12 ± 3	34 ± 4
	.01	17 ± 1	33 ± 12	80 ± 15	106 ± 35	24 ± 6	22 ± 7	14 ± 2	28 ± 4
Neutral	0	27 ± 4	32 ± 7	117 ± 6	112 ± 10	21 ± 2	26 ± 4	17 ± 2	21 ± 5
	1	179 ± 81	269 ± 97	147 ± 12	248 ± 40	26 ± 5	27 ± 13	16 ± 5	65 ± 11
	.5	100 ± 39	161 ± 28	123 ± 16	206 ± 18	33 ± 7	23 ± 7	16 ± 4	60 ± 4
	.1	50 ± 13	90 ± 26	114 ± 3	172 ± 18	37 ± 12	22 ± 4	13 ± 5	40 ± 6
	.05	43 ± 13	54 ± 8	120 ± 6	144 ± 29	39 ± 3	24 ± 5	17 ± 5	32 ± 8
	.01	33 ± 9	34 ± 17	130 ± 15	113 ± 12	33 ± 13	23 ± 5	19 ± 13	21 ± 1

* - NT = not tested.

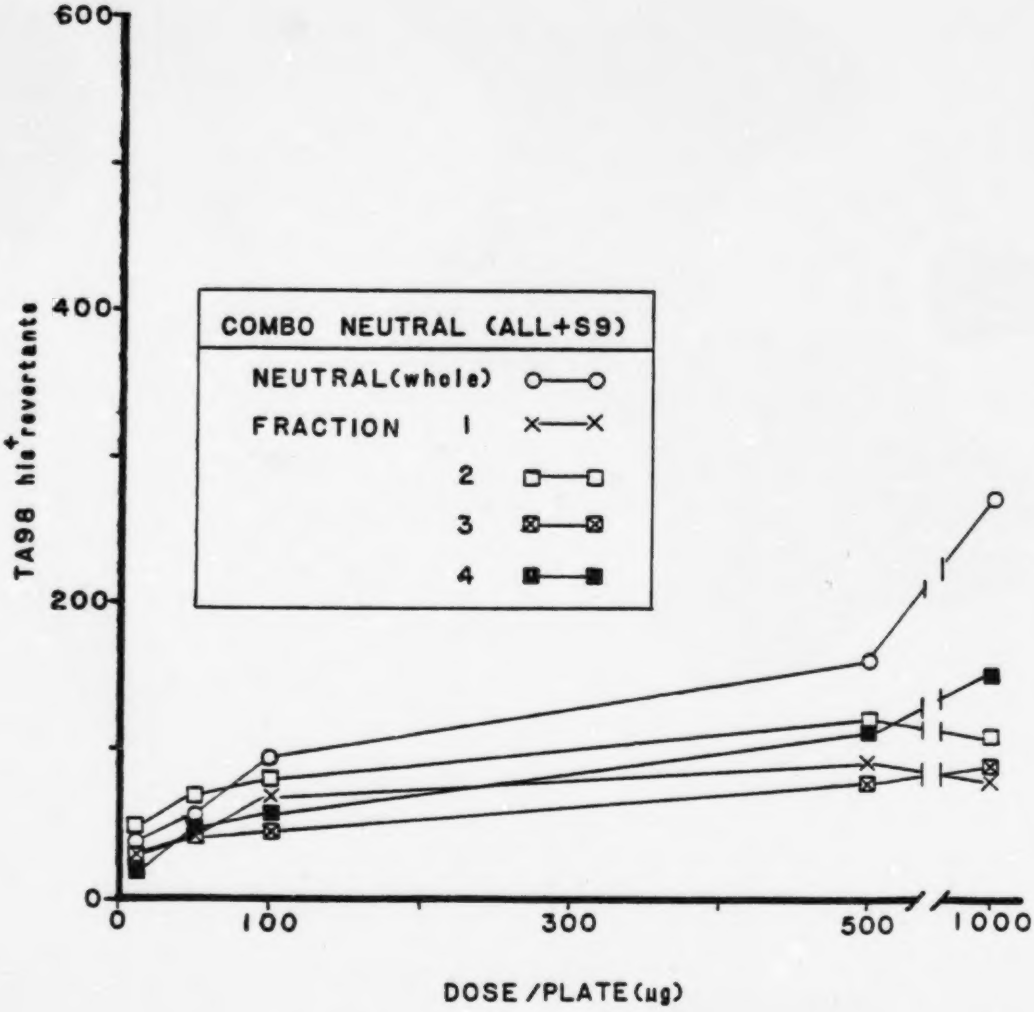


Figure 38. Mutagenic activity of subfractions of neutral fraction of COMBO waste.

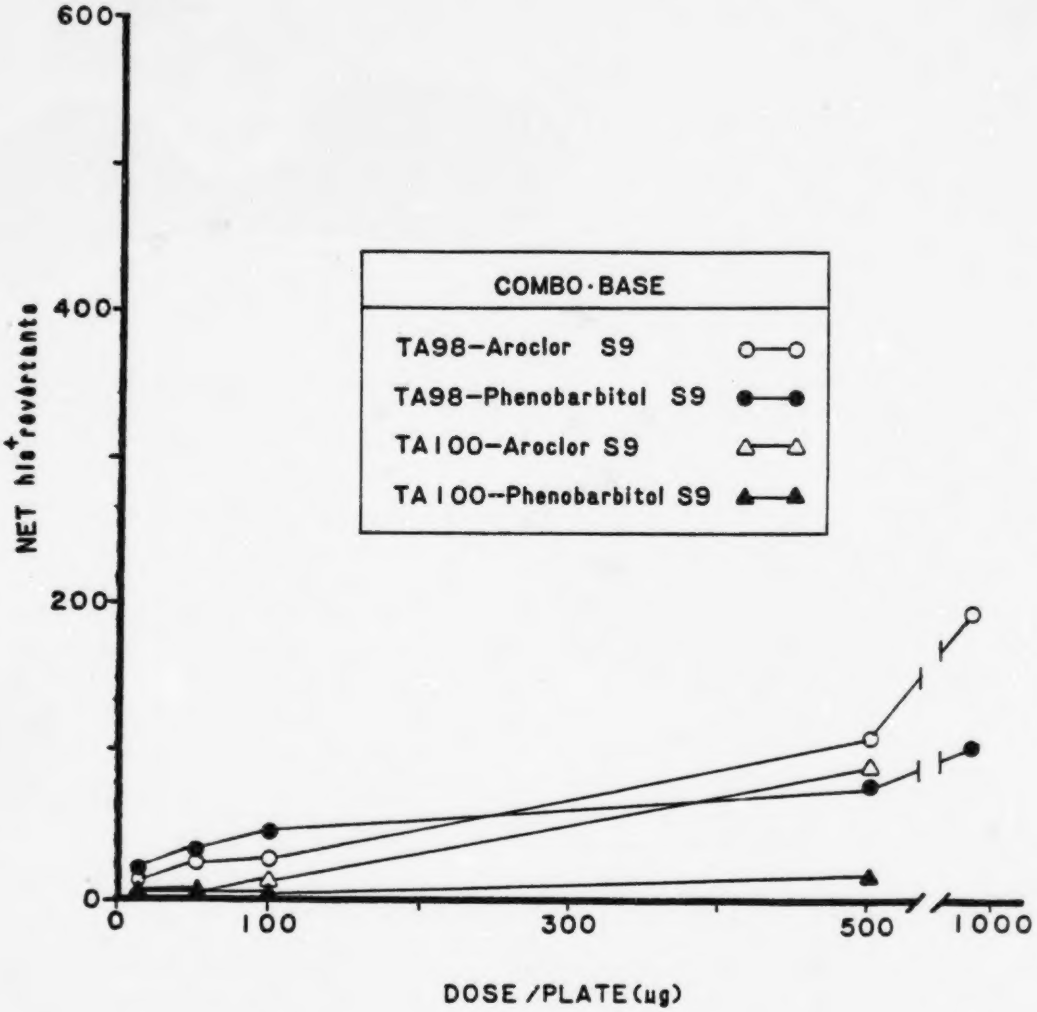


Figure 39. Mutagenic response of base fraction of COMBO waste using metabolic activation from Aroclor 1254 or phenobarbitol induced rat liver.

TABLE 37. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF A. NIDULANS FOLLOWING EXPOSURE TO ACID FRACTION OF COMBO WASTE

Dose/Plate (µg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
131.5	40	.62	2.2	.39	0	2.6
436.5	40	.28	25.4	0	0	25.4
657.5	40	.11	8.6	2.0	0	10.6
873	40	.09	69.6	0	0	69.6
1315	40	.08	10.0	0	0	10.0
With Metabolic Activation						
164	20	.59	4.0	3.7	1.0	8.7
436.5	20	.34	36.8	68.2	15.8	120.8
655	20	.15	12.9	24.9	5.9	43.7
873	20	.04	176.4	367.6	73.5	617.4
1315	20	.30	9.4	20.0	3.6	33.1

TABLE 38. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF *A. NIDULANS* FOLLOWING EXPOSURE TO BASIC FRACTION OF COMBO WASTE

Dose/Plate (µg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
130	40	.75	3.2	.29	0	3.5
221	40	.69	6.0	.89	0	6.9
275	40	.49	6.0	1.8	0	7.8
441	40	.37	12.6	1.6	0	14.2
550	40	.34	9.0	3.1	0	12.0
With Metabolic Activation						
110	20	.83	4.7	1.7	.66	7.1
221	20	.66	7.0	6.7	3.7	17.4
330	20	.48	8.3	5.8	1.4	15.5
441	20	.43	22.7	19.1	8.6	50.4
550	20	.39	7.9	8.8	1.8	18.5

TABLE 39. SURVIVING FRACTION AND INDUCED MUTATION FREQUENCY OF A. NIDULANS FOLLOWING EXPOSURE TO NEUTRAL FRACTION OF COMBO WASTE

Dose/Plate (µg)	Exposure Time (min)	Surviving Fraction	Mutation Frequency Per 10^6 Survivors			
			A	B	C	Total
Without Metabolic Activation						
85	40	.87	3.9	3.4	0	7.3
171	40	.69	7.7	6.9	0	15.4
214	40	.46	8.6	8.2	0	16.8
320	40	.43	15.0	13.7	0	28.7
428	10	.82	1.6	2.4	0	4.0
550	40	.30	17.1	15.7	0	32.8
With Metabolic Activation						
106.9	20	.75	2.0	.76	.68	3.4
213.8	20	.38	5.4	2.8	1.4	9.6
221.0	20	.34	36.7	68.2	15.8	120.7
427.5	20	.19	34.7	39.0	13.0	86.7
441	20	.04	176.3	367.6	73.5	617.4

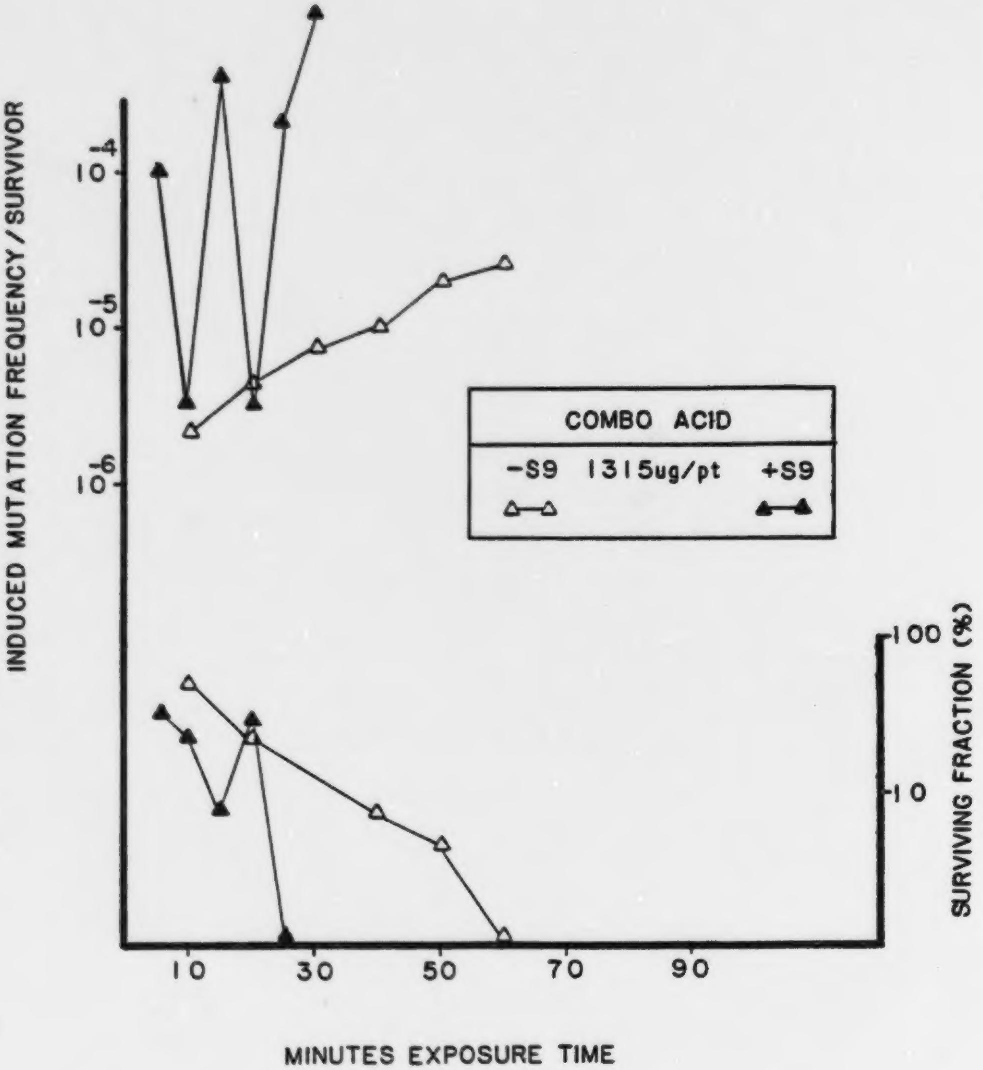


Figure 40. Induced mutation frequency and fractional survival in *A. nidulans* following exposure to acid fraction of COMBO waste. Data based on results from two independent experiments.

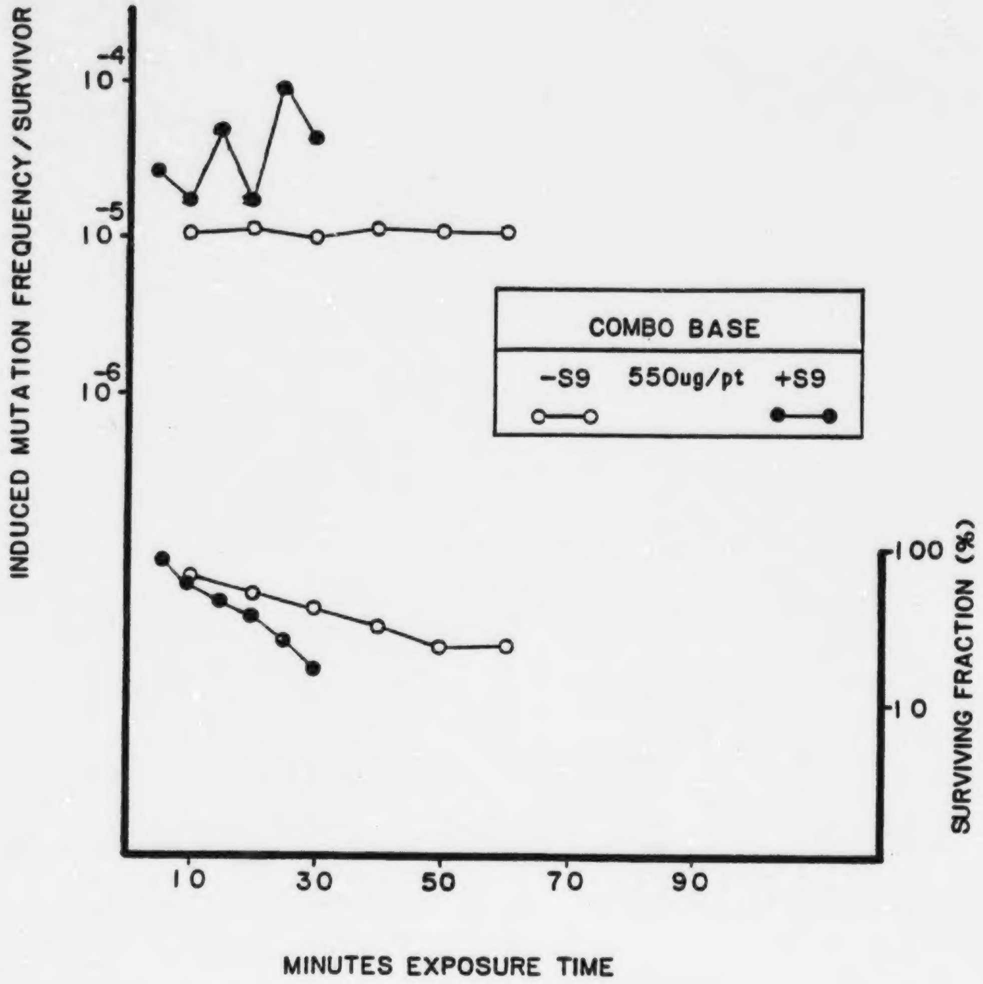


Figure 41. Induced mutation frequency and fractional survival in A. nidulans following exposure to base fraction of COMBO waste. Data based on results from two independent experiments.

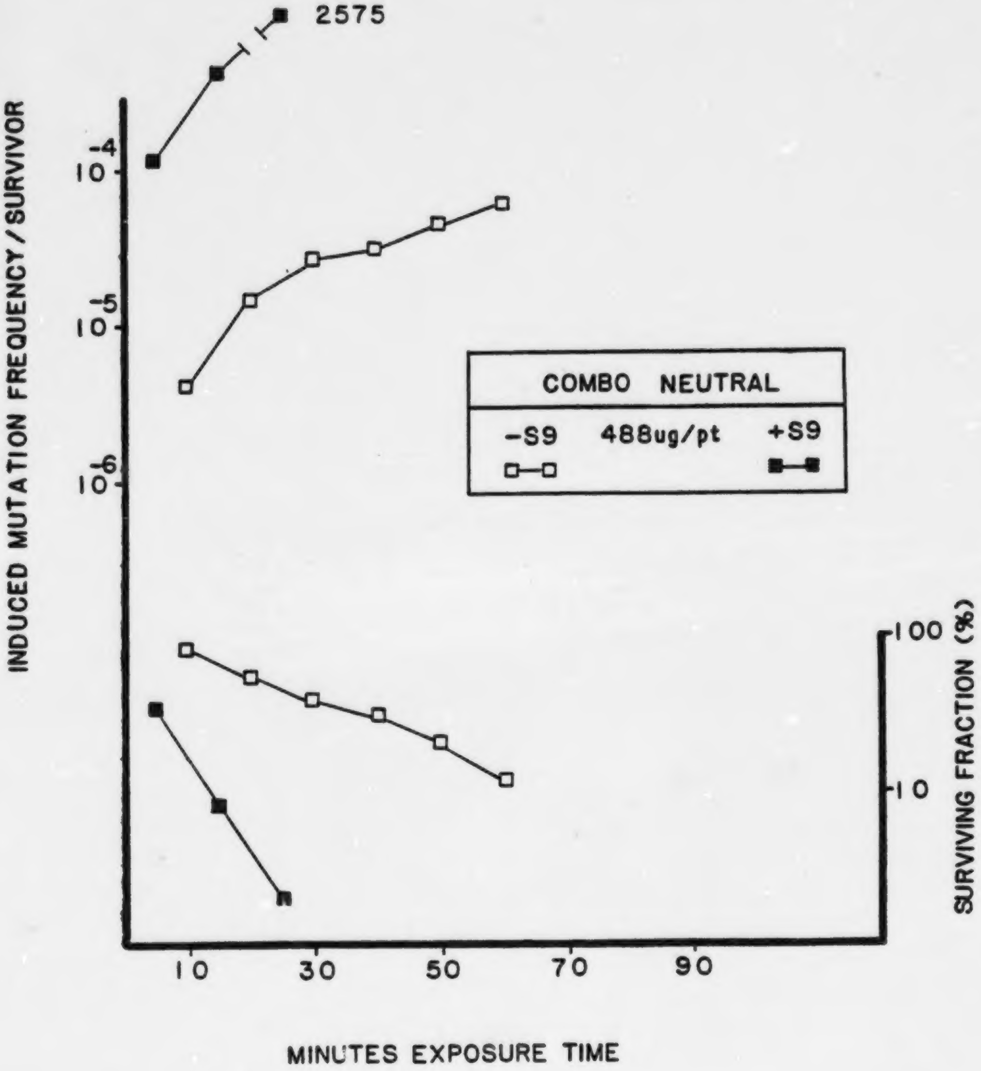


Figure 42. Induced mutation frequency and fractional survival in A. nidulans following exposure to neutral fraction of COMBO waste. Data based on results from two independent experiments.

acid, base and neutral fractions of the COMBO waste were .049, .70, and .057, respectively (Table 29). The induced segregation index with respect to DMSO was 1.14 for the acid, 1.63 for the base, and 1.33 for the neutral fraction (Table 29). The acid and base fractions induced the maximum mutation frequency in the hyperdiploid class, while the neutral fraction induced the maximum mutation frequency in the class representing mitotic recombination. In addition, the acid fraction induced a significant increase in the breakdown of the mitotic spindle apparatus. These results indicate that in abnormal colonies the maximum genotoxic response was induced by the base fraction, with the neutral and acid fractions inducing slightly lower responses. Thus, in both the haploid and diploid *Aspergillus* assays, the maximum overall genotoxic response was obtained with the neutral fraction, with an intermediate response in the acid fraction, and a borderline response in the base fraction.

Chemical analysis identified a total of eleven, seven, and eight compounds in the acid, base, and neutral fractions, respectively, of the COMBO waste (Figures 43, 44, 45, and Table 40). In all three fractions, at least half of the compounds identified were substituted napthalenes. Only one compound, dimethyl phenanthrene,identified in the COMBO waste fractions would be considered a potential source of mutagenic activity, and it was identified in the neutral fraction. A study by LaVoie *et al*. (1983) determined that several substituted phenanthrenes were mutagenic in *Salmonella* with metabolic activation.

Bioassay directed chemical analysis of the fractions of the combined API separator/slop-oil emulsion waste detected compound(s) capable of inducing point mutations, lethal damage to DNA, and various types of chromosome damage. Toxic effects were not observed in the prokaryotic point mutation assay even at the highest dose levels. The maximum genotoxic response in the *Salmonella*, *Aspergillus* haploid, and *Aspergillus* diploid bioassays was induced by the neutral fraction; while the acid fraction induced the maximum rsponse in the *Bacillus* DNA repair assay. The neutral fraction induced a significant mutagenic response both with and without metabolic activation. This fraction also contained dimethyl phenanthrene, the only potential mutagen identified in the COMBO waste. Substituted phenanthrenes, however, are known to require metabolic activation in order to reach their ultimate mutagenic form (LaVoie *et al*., 1983). Thus, these results indicate that additional compound(s), not identified by chemical analysis and present in the COMBO waste, are likely to be responsible for the direct-acting mutagenic activity detected in the neutral fraction and for the indirect-acting mutagenic activity of the other waste fractions. Both the chemical and the biological analysis of the COMBO waste indicated that the neutral fraction contained the maximum mutagenic potential. The constituent most likely to be responsible for the mutagenic activity of the neutral fraction, dimethyl phenanthrene, was detected by a chemical analysis. However, land application of the COMBO waste should be monitored using a combined testing protocol because biological

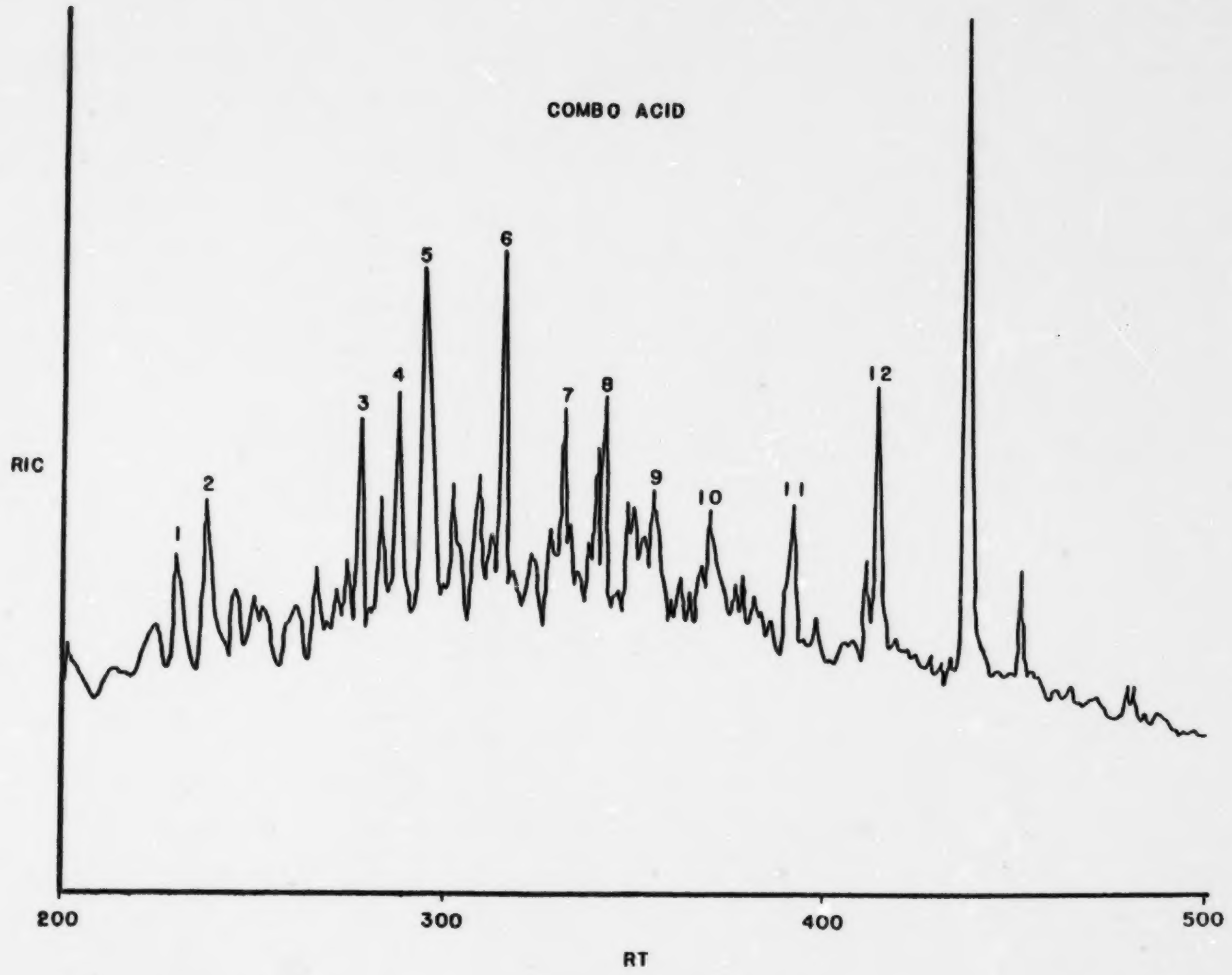


Figure 43. GC/MS chromatograph of acid fraction of COMBO waste.

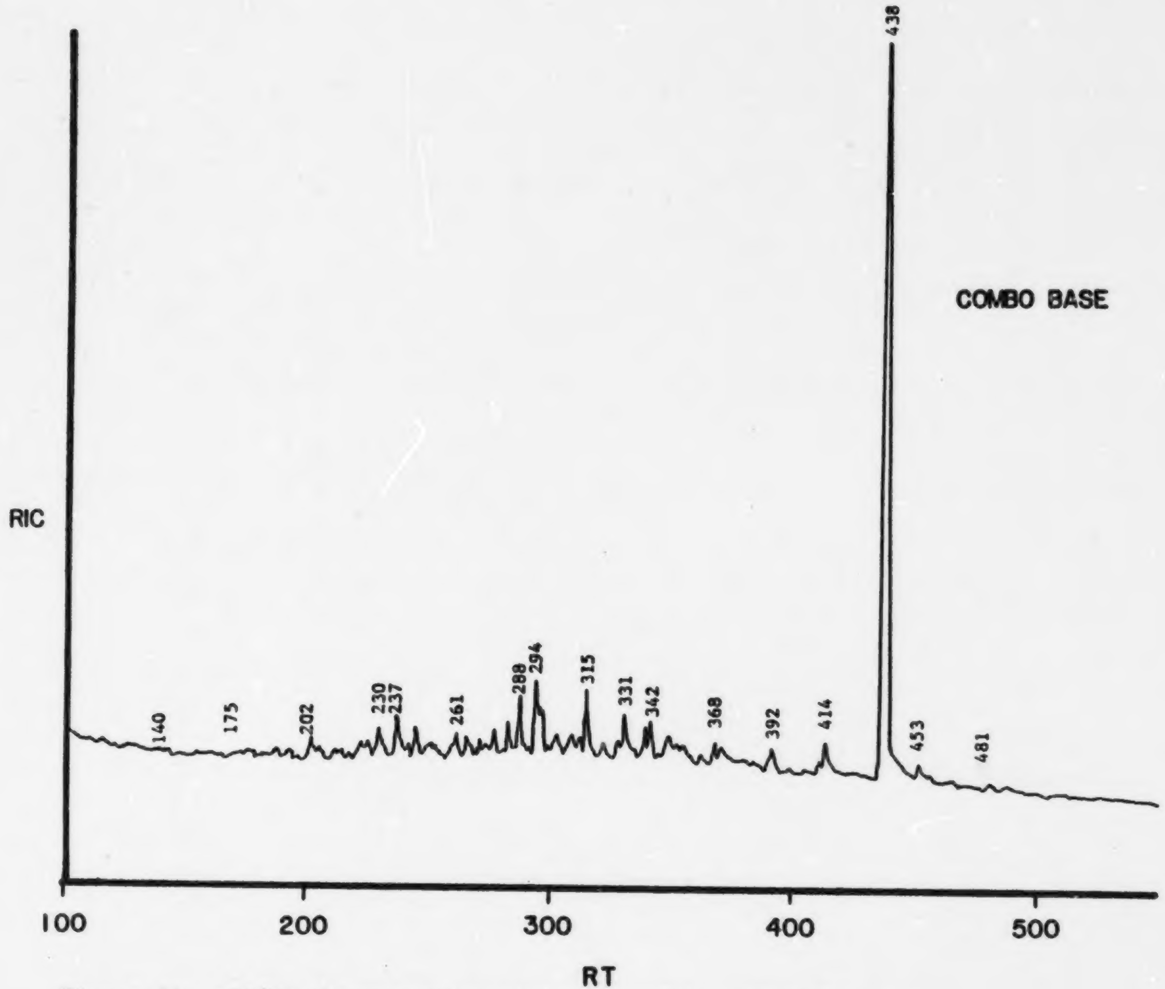


Figure 44. GC/MS chromatograph of base fraction of COMBO waste.

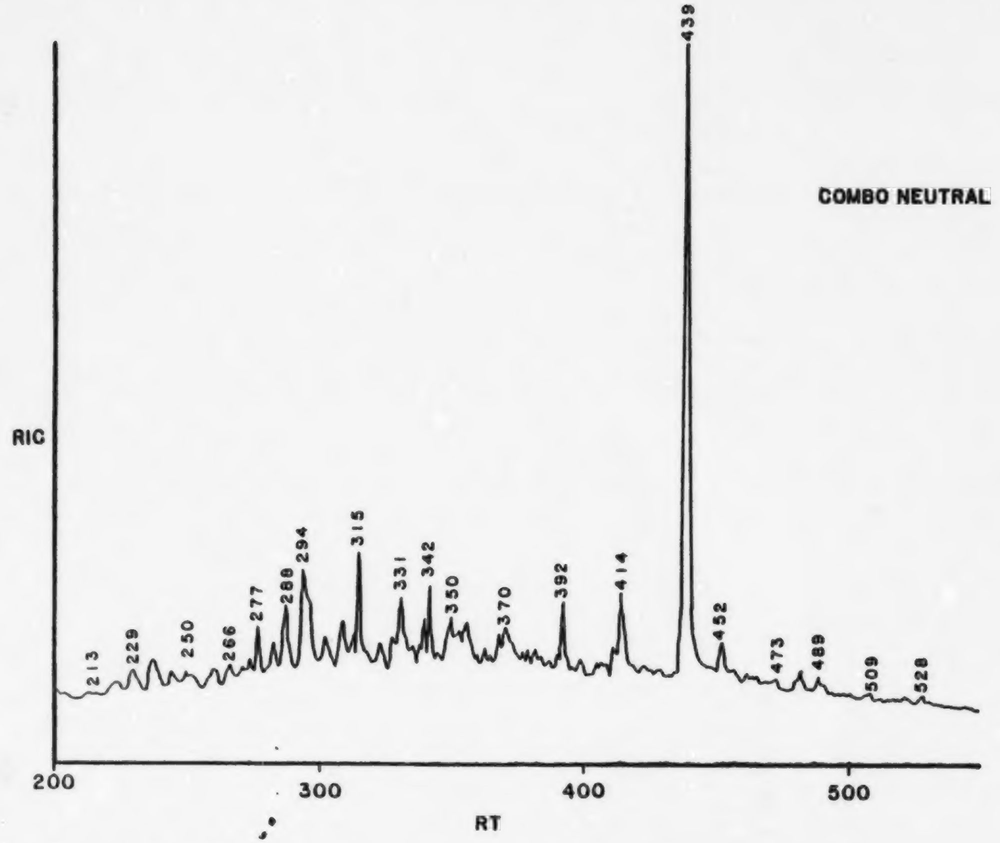


Figure 45. GC/MS chromatograph of neutral fraction of COMBO waste.

TABLE 40. SELECTED PROPERTIES OF COMPOUNDS IDENTIFIED IN THE FRACTIONS OF THE COMBINED API-SEPARATOR/SLOP-OIL EMULSION SOLIDS WASTE

Sample	Peak Number	Compound	CAS #	Formula	Genetic Activity
Acid Fraction	229	Trimethyl octane	-	$C_{11}H_{24}$	X
	237	Methyl napthalene	90-12-0	$C_{11}H_{10}$	M1;CO
	277	Ethyl-methyl octane	-	$C_{11}H_{24}$	X
	287, 294	Dimethyl napthalene	573-98-8	$C_{12}H_{12}$	MO
	315	Trimethyl decane	-	$C_{13}H_{28}$	X
	331	Methyl ethyl napthalene	-	$C_{13}H_{14}$	X
	342	Trimethyl napthalene	879-129	$C_{13}H_{14}$	MO
	392	Dimethyl undecane	-		X
	414	Hexyl tridecane	-		X
Basic Fraction	230	Trimethyl octane	-	$C_{11}H_{24}$	X
	237	Methyl napthalene	90-12-0	$C_{11}H_{10}$	M1;CO
	288	Dimethyl napthalene	573-98-8	$C_{12}H_{12}$	MO
	315	Trimethyl decane	-	$C_{13}H_{28}$	X
	331	Methyl ethyl napthalene	-	$C_{13}H_{14}$	X
	342	Trimethyl napthalene	879-129	$C_{13}H_{14}$9	X
Neutral Fraction	277	Ethyl-methyl octane	-	$C_{11}H_{24}$	X
	287, 294	Dimethyl napthalene	573-98-8	$C_{12}H_{12}$	M1;CO
	315	Trimethyl octane	-	$C_{11}H_{24}$	X
	331	Methyl ethyl napthalene	-	$C_{13}H_{14}$	X
	342	Trimethyl napthalene	879-129	$C_{13}H_{14}$	MO
	392	Dimethyl undecane	-	$C_{13}H_{28}$	X
	528	Dimethyl phenanthrene	-	$C_{16}H_{14}$	M1;C1

1. Potential genetic activity: P = promotor; I = inhibitor; CC = cocarcinogenic; M1 = mutagenic; MO = nonmutagenic; C1 = carcinogenic; CO = noncarcinogenic; X = unknown; references included in text.

analysis detected trace quantities of additional genotoxic compounds not identified by a chemical analysis.

Biological and chemical analysis have been employed in the present study to evaluate the mutagenic potential of the acid, base, and neutral fractions of three hazardous industrial wastes. A summary of the results obtained in the different biological test systems is provided in Table 41. The base fraction of the wood-preserving waste induced the maximum mutagenic response in the *B. subtilis* DNA repair plate incorporation assay, the *Salmonella*/microsome assay (strains TA98, TA100 and TA1538) and the *Aspergillus* methionine assay. In the DNA repair spot test and *Aspergillus* diploid assay, the acid fraction of the wood-preserving waste induced the maximum response. One component of the acid fraction which may be responsible for its genetic toxicity is pentachlorophenol (Fishbein, 1979). Pentachlorophenol does not induce a mutagenic response in the *Salmonella* assay (Anderson *et al*., 1972). However, in the recently developed *S. typhimurium* strain TA102, the maximum response was induced by the acid fraction. The results of biological analysis indicate that all three fractions of the wood-preserving waste contain genotoxic compounds, while chemical analysis identified compounds that may be mobile and resistant to degradation. Thus, land treatment of a wood-preserving waste should be carefully monitored and preferrably conducted at low application rates. Although chemical analysis failed to identify any of the constituents of the storm-water runoff impoundment, biological analysis detected mutagenic activity in all three waste fractions. The maximum mutagenic response observed in three of four bioassays was induced by the acid fraction of the SWRI waste. Biological analysis detected genotoxic compounds in the SWRI waste; however, these compounds were present in quantities that were below the detection limits of the chemical analysis. Thus, the storm-water runoff impoundment may be a good candidate for land treatment because of its relatively low concentration of mutagenic compounds.

Chemical and biological analysis agreed that the neutral fraction of the combined API separator/slop-oil emulsion solids possessed the maximum mutagenic potential. In the COMBO waste, biological and chemcial analysis identified the fraction with the greatest mutagenic potential, although chemical analysis was unable to identify all of the mutagenic constituents. These results indicate that for both refinery wastes, biological analysis may provide the most efficient tool for monitoring a land treatment facility.

This research has demonstrated the utility of a combined testing protocol using biological analysis to measure the genotoxic potential of waste fractions and a chemical analysis to define the type and quantity of genotoxic compounds. These results have also demonstrated the inability of chemical analysis to provide a comprehensive evaluation of the genotoxic potential of a hazardous industrial waste. While it is possible that a more intensive chemical analysis could have identified

TABLE 41. SUMMARY OF RESULTS OBTAINED FROM TESTING WASTE FRACTIONS IN BIOLOGICAL TEST SYSTEMS

Sample		Bioassay[1,2]									
		DNA		SALM		BAC PM		ASPMT		ASPDP	
	S9[3]	−	+	−	+	−	+	−	+	−	+
PENT S	Acid	+	+	−	+	−	+	+	++	+	0
	Base	−	++	−	++	−	+	+	++	±	0
	Neutral	−	−	−	+	−	−	+	+	+	0
SWRI	Acid	−	−	−	++	0	0	+	++	+	0
	Base	−	−	−	+	0	0	+	++	+	0
	Neutral	−	−	−	++	0	0	+	+	+	0
COMBO	Acid	−	+	−	++	0	0	+	+	+	0
	Base	−	−	−	++	0	0	+	+	±	0
	Neutral	−	−	+	++	0	0	+	++	+	0

1. DNA = *B. subtilis* DNA repair assay; SALM = *S. typhimurium* reverse mutation assay; BAC PM = *B. subtilis* reverse mutation assay; ASPMT = *A. nidulans* methionine assay; ASPDP = *A. nidulans* diploid assay.
2. Response: 0 = not tested; − = <2 times background; ± = >2 <2.5 times background; + = >2.5 <5 times background; + + = ≥5 times background.
3. S9 = 9000 x g supernatant from Aroclor 1254 induced rats.

genotoxic compounds present in trace concentrations, information would still be lacking as to the interactions of the waste constituents. However, the results also indicate that chemical analysis is a necessary component of a hazardous waste analytical protocol. Chemical analysis is the only procedure that can be used to identify waste constituents and to verify the absence of artifacts generated in the collection or extraction process.

SECTION 6

SOIL CHARACTERIZATION

INTRODUCTION

As the products of our industrialized society become more complex, the need correspondingly increases to develop the technology that can provide for the safe disposal of the waste. The use of soil to receive and degrade waste materials has gained general acceptance while other methods of waste disposal have become more restricted for economical or environmental reasons. In order to prevent further loss of an already diminishing natural resource, land disposal of waste should be managed in order to provide the most efficient use of the land and monitored in order to evaluate the progress of waste degradation. Land treatment is a method of waste disposal that is designed to utilize the diverse microbial population of a fertile soil for the degradation of waste constituents, while the adsorptive capacity of the soil prevents environmental deterioration. EPA (1982) regulations require that land treatment be restricted to wastes that are rendered less or non-hazardous through chemical and biological reactions in the soil. In order to provide information that can demonstrate the effect of soil incorporation on the hazardous characteristics of a waste, techniques need to be developed for monitoring the mutagenic potential of environmental samples before and after waste application.

Short-term bioassays have been proposed as a tool for monitoring land treatment because biological analysis can often be used to detect the synergistic, antagonistic, or additive interactions of the components of a complex mixture. The first step in establishing a land treatment program is to demonstrate that the waste is rendered less or non-hazardous by soil incorporation. This treatment demonstration should be preceded by an evaluation of the mutagenic potential of both the waste and the receiving soil. This study reports on the results of a mutagenic potential evaluation of the organic extract of three native soils used solely for agricultural purposes. These procedures may also prove useful for evaluating the point of sufficient reclamation for a soil contaminated by a chemical spill.

MATERIALS AND METHODS

Soil

Three soils were selected to represent a range in soil texture and management practices. Physical characteristics of these soils are given in Table 42. A Norwood sandy clay (Typic Udifluvent) was obtained from the Texas A&M University Research Farm. This soil had been fallow for four to five years prior to being cropped to a sorghum-sudan hybrid. The Norwood soil has a Class I land use capability which indicates this series has very few limitations and can be used for intensive cultivation practices. Potential sources of mutagenic contaminants in the Norwood soil include exhaust from the tractors and direct application or drift of biocides from sprayed crops. The Bastrop clay (Udic Paleustalf) was collected from a range area being used for cattle grazing at the Texas A&M University Research Annex. The soil series at this site consisted of a well-drained fine sandy loam surface soil approximately 45 cm thick and underlain by a massive clay loam subsoil. Because of the limited permeability of the subsoil, the Bastrop series is primarily used as rangeland and is rarely cultivated. The range area had been sprayed with a herbicide mixture of 2,4-D and 2,4,5-T in July and August preceeding the October collection of soil. Dr. W. D. Burge supplied a sample of Sassafrass sandy loam (Mesic Typic Hapludult) from the Beltsville Agricultural Research Center. This soil had been fallow for about five years until being cropped to corn (*Zea mays*) and fertilized during the three years prior to soil collection. Herbicide treatments that had been applied at acceptable rates to the Sassafras soil included atrazine, lasso, simazine, paraquat, carbofuran, and toxaphene. Hydrocarbons were extracted from the soil using dichloromethane following the methods of Brown and Donnelly (1983). Solvent extractions were taken to dryness on a Brinkman Bucci Rotary Evaporator, and the residue was dissolved in dimethyl-sulfoxide (Grade 1, Sigma Chemical Co., St. Louis, MO) for testing in the biological systems.

Biological Analysis

The ability of the organic extract of soil samples to induce genetic damage was measured in a prokaryotic and eukaryotic system capable of detecting compounds that induce point mutations. The *Salmonella*/microsome assay of Ames *et al*. (1975) utilizes a prokaryotic organism to evaluate the capacity of a sample for inducement of reverse mutations to histidine prototrophy. The two *Salmonella* strains TA98 (a frameshift mutant) and TA100 (a base-pair substitution mutant) were supplied by Dr. B. N. Ames (University of California, Berkeley, CA). The methods used were the same as Ames *et al*. (1975) except that overnight cultures were prepared by inoculation into 10 ml of Oxoid Nutrient Broth No. 2 (K C Biological, Lenexa, KS). Soil extracts were tested on duplicate plates in two independent experiments in the standard plate incorporation assay at a minimum of four dose

TABLE 42. PHYSICAL PROPERTIES OF THE THREE SOILS

Soil	Sand	Silt	Clay	Texture	pH	Extractable Hydrocarbon (%)
	(%)					
Bastrop	60.3	10.0	29.7	SCL	6.9	.229
Norwood	48.2	15.2	36.6	SC	7.7	.057
Sassafrass	57.27	34.86	7.87	SL	5.6	.025

levels with and without enzyme activation (0.3 ml rat liver/ml S.9 mix). Aroclor 1254 induced rat liver was obtained from Litton Bionetics (Charleston, SC). Positive controls included 2 µg/plate N-methyl-N -nitro-N-nitrosoguanidine (Sigma) for TA100, 25 µg/plate 2-nitrofluorene, (Aldrich Chemical Co., Milwaukee, WI) for TA98, and 10 µg/plate 2-aminoanthracene (Sigma) which was used to verify the functioning of the metabolic activation system. All reagents and extracts were tested for sterility; dimethylsulfoxide was used as a negative control.

The eukaryotic test employed Aspergillus nidulans (a fungus) to assess the mutagenic potential of soil extracts by evaluating the induction of foreward mutations at the methionine suppressor loci. Conidia from four to five single colonies of the methGl biAl (requiring methionine and biotin) Glasgow strain of Aspergillus nidulans grown for 5-6 days on a complete medium at 37°C were used for each experiment. Samples were tested at a minimum of five dose levels and four exposure times with and without metabolic activation. The procedures used were the same as Scott et al. (1978). Mutant colonies were assayed by spreading exposed cells on a methionine-free medium. Mutant colonies were scored after incubation for 5 days at 37°C. Colonies were divided by colony morphology into three Classes, A, B, C, and the total number of mutant colonies. Each of the three classes is believed to involve two genes (Scott and Alderson, 1970). The morphology of Class A colonies appear green, Class B brown, and Class C green with a white hyaline edge. The frequency of mutations induced by the soil extract was determined by subtracting the frequency of spontaneous mutations which occurred in Class A, B, C or the total from the total mutation frequency in Classes A, B, C and the total. A sample was considered positive if a positive slope occurred on the mutation induction curve, or the induced mutation frequency for at least two exposure times was more than twice the spontaneous mutation frequency. Positive controls included 8-methoxypsoralen (Sigma), 8-methoxypsoralen plus near UV-light without activation, and benzo(a)pyrene (Aldrich) with metabolic activation. Only the Bastrop and Norwood soil extracts were tested in the Aspergillus assay.

RESULTS AND DISCUSSION

The results from testing each of the three soils in the Salmonella/microsome assay (Table 43, Figures 46 and 47) indicate that all soil extracts contained mutagenic activity, with the greatest amount of activity being detected in the Bastrop soil. In strain TA98 with metabolic activation, 1,000 µg of the extract of the Norwood, Sassafrass, and Bastrop soils induced 67, 119, and 469 revertant colonies, respectively. The mutagenic activity of the organic extract from one gram of the Bastrop soil was greater than the Norwood or Sassafrass soils by almost two orders of magnitude and less than the condensate from the smoke of one cigarette by one order of magnitude (Figure 48).

TABLE 43. MUTAGENICITY OF ORGANIC EXTRACTS OF THREE SOILS AS MEASURED WITH S. TYPHIMURIUM STRAINS TA98 AND TA100 WITH AND WITHOUT METABOLIC ACTIVATION

Soil	Dose/plate (μg)	Total his Revertants (Mean + SD)			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Norwood	0	26 ± 6.9	32 ± 7.7	88 ± 14	130 ± 27
	10	23 ± 3.3	38 ± 9.2	76 ± 9.7	122 ± 49
	50	NT*	NT	NT	NT
	100	27 ± 6.9	33 ± 11	87 ± 15	107 ± 35
	500	NT	NT	NT	NT
	1000	32 ± 8.3	67 ± 16	116 ± 19	140 ± 8.1
	5000	51 ± 23	122 ± 45	88 ± 14	188 ± 52
Sassafrass	0	19 ± 3.1	21 ± 5.3	106 ± 16	117 ± 17
	10	25 ± 5.0	30 ± 7.6	115 ± 11	146 ± 20
	50	37 ± 6.2	42 ± 4.9	172 ± 43	147 ± 25
	100	31 ± 21	36 ± 6.7	185 ± 80	117 ± 18
	500	59 ± 10	61 ± 5.6	251 ± 78	186 ± 28
	1000	69 ± 14	199 ± 35	265 ± 115	288 ± 65
	2500	112 ± 20	179 ± 16	276 ± 18	NT
Bastrop	0	23 ± 5.8	35 ± 6.7	124 ± 22	94 ± 12
	10	28 ± 0.8	48 ± 6.6	121 ± 23	101 ± 15
	50	47 ± 8.0	62 ± 3.2	143 ± 56	141 ± 27
	100	183 ± 81	95 ± 29	142 ± 50	142 ± 52
	500	256 ± 22	264 ± 90	306 ± 33	381 ± 119
	1000	423 ± 34	469 ± 96	416 ± 13	429 ± 74
	5000	515 ±256	716 ± 108	605 ± 72	NT

*NT - Not tested.

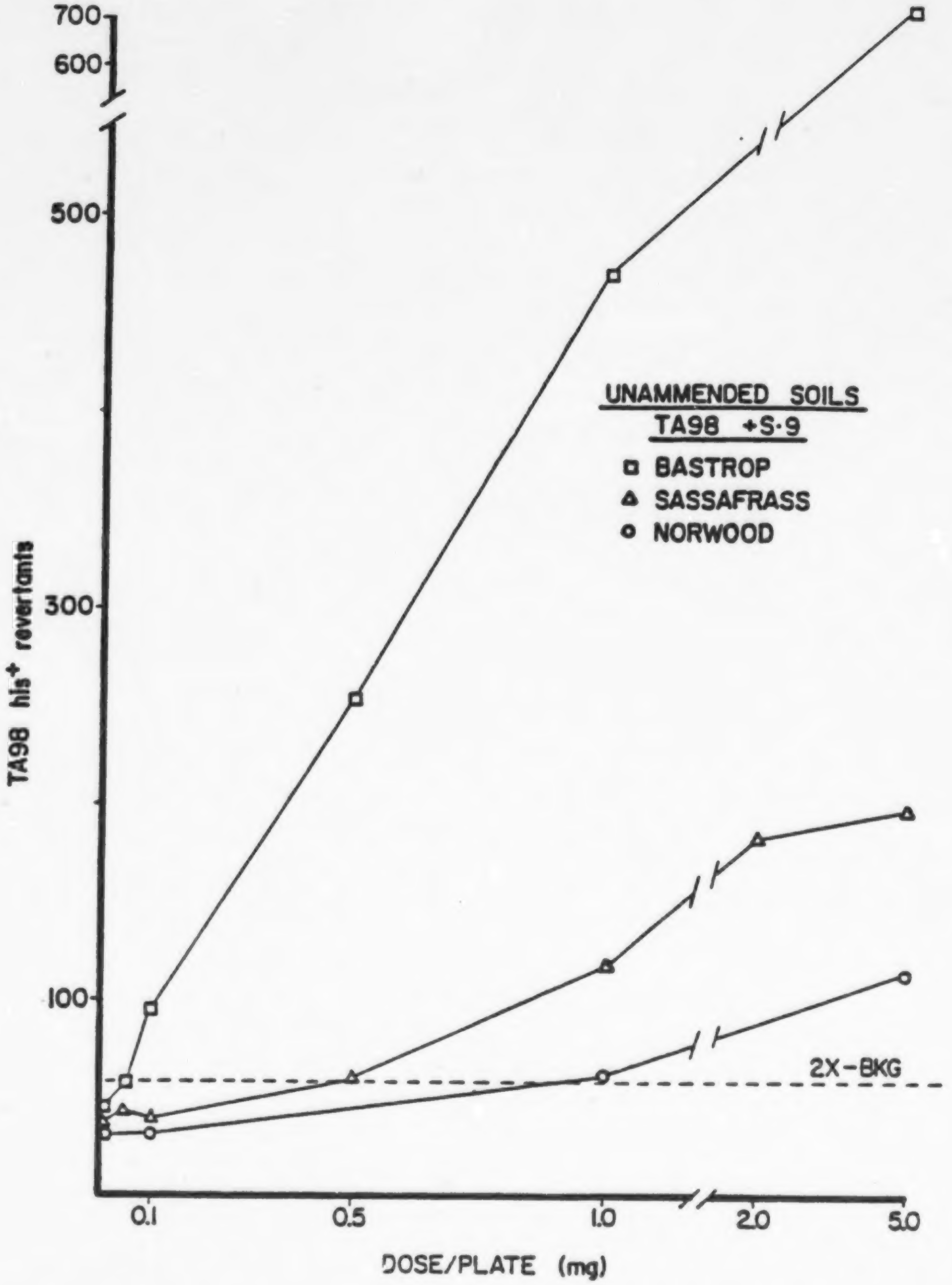


Figure 46. Mutagenic activity of organic extract of three agricultural soils as measured with S. typhimurium, strain TA98, with metabolic activation.

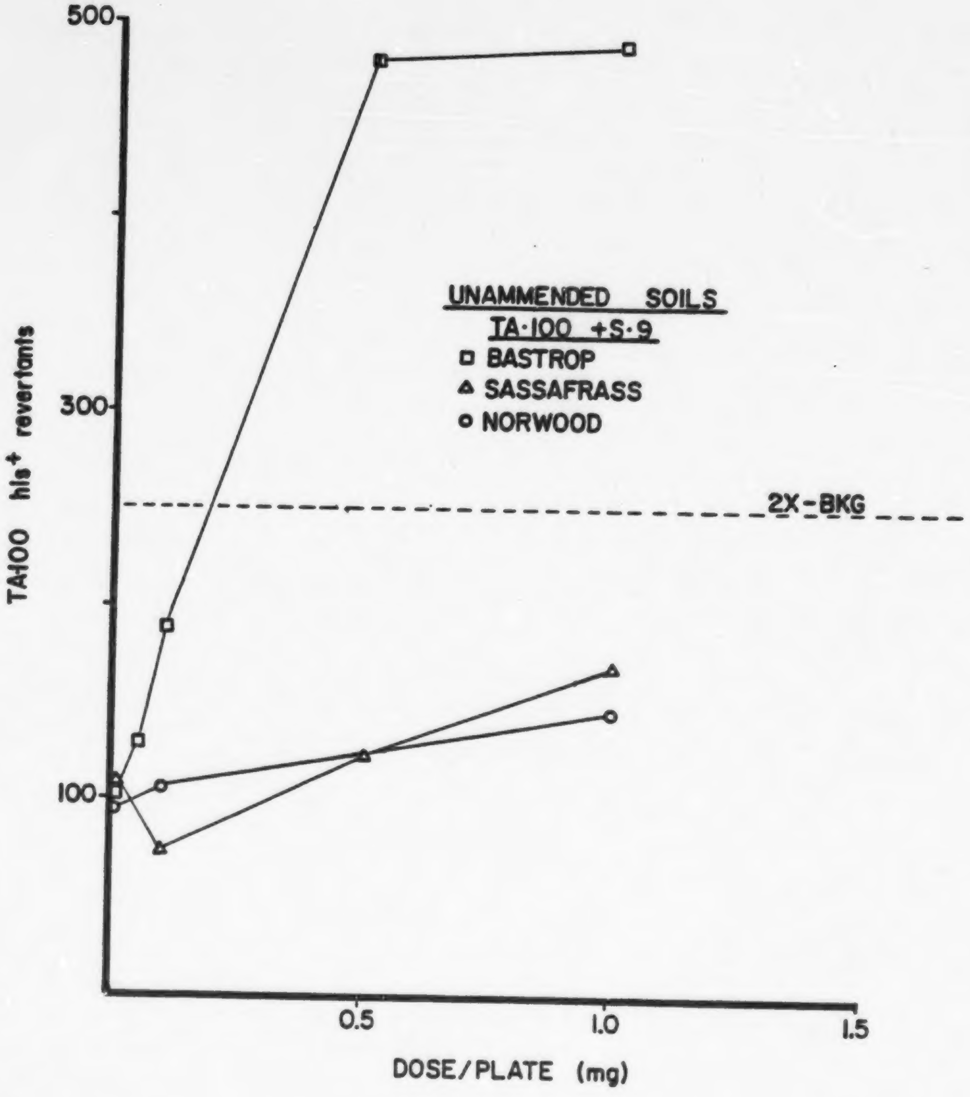


Figure 47. Mutagenic activity of organic extract of three agricultural soils as measured with S. typhimurium, strain TA100, with metabolic activation.

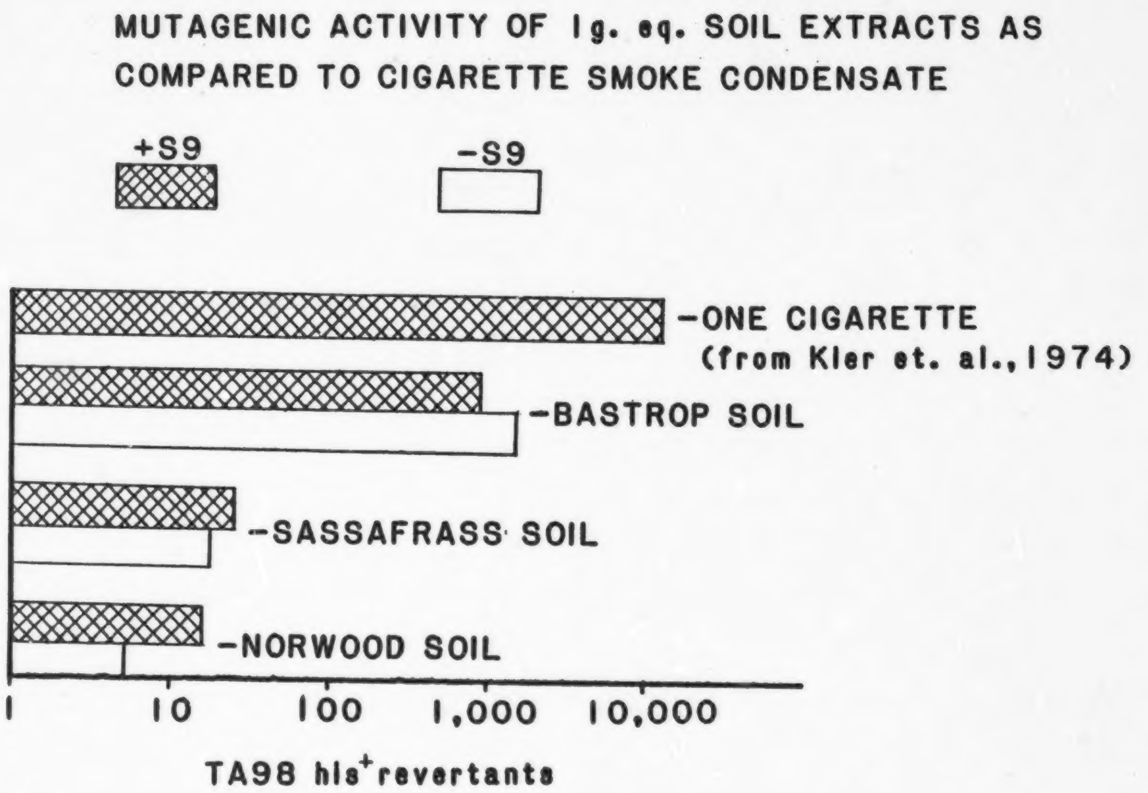


Figure 48. Mutagenic activity of one gram of soil as compared to cigarette smoke condensate.

The dose-response curves for all three soils in strain TA98 with and without metabolic activation (Figures 46 and 49) indicate that while direct acting mutagens were detected in the Sassafrass and Bastrop soils, the addition of metabolic activation to the assay system increased the number of revertant colonies. The addition of metabolic activation produced almost twice the number of revertant colonies obtained in the absence of metabolic activation in both the Sassafrass and Norwood soils in strain TA98 at the highest dose levels. For the Bastrop soil, there was only a slight increase in the total number of revertant colonies when metabolic activation was added to the assay system (Table 43).

The organic extracts of the Bastrop and Norwood soil were also tested in an eukaryotic bioassay using *A. nidulans*. This bioassay has been found to detect 95% of carcinogens as mutagens and is sensitive to some compounds not detected in the *Salmonella* assay (Scott *et al.*, 1982). The results presented in Table 44 and Figures 50 and 51 indicate that the organic extract of the Bastrop and Norwood soils induced increased mutation frequencies with increasing exposure times in the absence of metabolic activation. The frequencies of mutations induced by the two soils were comparable, although the Norwood soil was tested at a dose level ten times greater than the Bastrop soil. For both soils, survival of *Aspergillus* in the absence of metabolic activation was decreased as the exposure time or dose level was increased. The effect of increasing dose level on the frequency of induced mutations was less conclusive. In the Norwood soil, an increased mutation frequency was observed as the dose level was increased, while this effect in the Bastrop soil was not observed.

When metabolic activation was added to the system, the response obtained in the *Aspergillus* assay was much less conclusive (Table 44 and Figures 50 and 51). Survival of *Aspergillus* was not appreciably altered at increased dose levels or exposure times. In addition, the frequency of mutations induced by the extract of either soil was not significantly affected by increasing the dose level or exposure time in the presence of metabolic activation. Thus, while mutagenic activity was detected in the organic extract of the two soils in the absence of metabolic activation, the addition of metabolic activation reduced or eliminated this effect.

An analysis of the extracts of the two soils using gas chromatographic and mass spectrometric techniques detected the presence of several low molecular weight chemicals. A total of thirteen compounds were identified in the Norwood soil and eight compounds in the Bastrop soil (Table 45). The chromatographs of the Norwood and Bastrop soils are provided in Figures 52 and 53, respectively. All thirteen compounds identified in the Norwood soil are saturated alkanes ranging from C_{14} (tetradecane) to C_{22} (docosane). Eight compounds were identified in the Bastrop soil including eight alkanes, four of which were saturated n-alkanes. The three additional compounds identified in the Bastrop

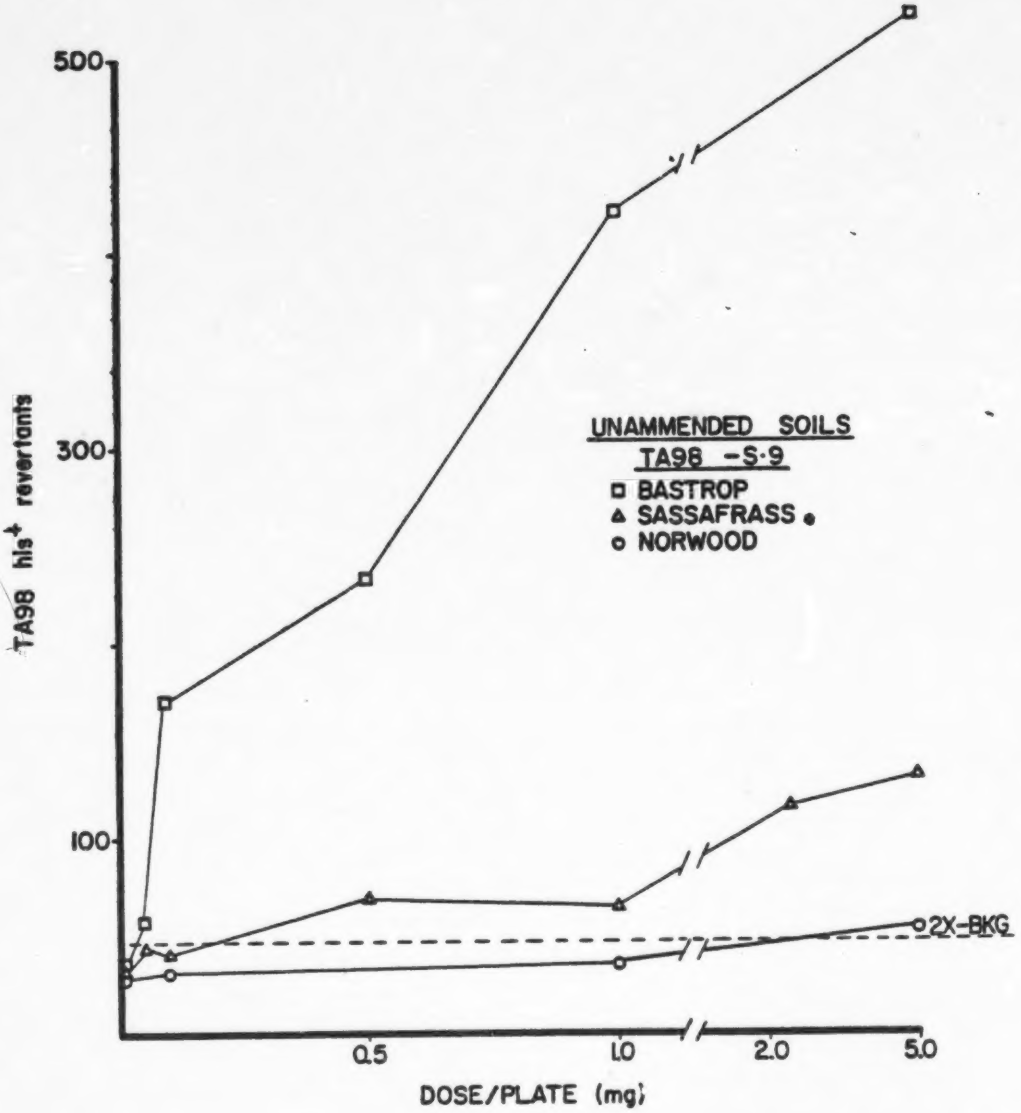


Figure 49. Mutagenic activity of organic extract of three agricultural soils as measured with *S. typhimurium*, strain TA98, without metabolic activation.

TABLE 44. EFFECT OF INCREASING DOSES OF ORGANIC EXTRACT OF BASTROP AND NORWOOD SOILS ON SURVIVAL AND INDUCED MUTATION FREQUENCY IN *ASPERGILLUS NIDULANS* WITH AND WITHOUT METABOLIC ACTIVATION

Dose/Plate (µg)	-S9 (Exposure Time - 40 min		+S9 (Exposure Time - 20 min)	
	Surviving Fraction	Induced Mutation Frequency (x 10^{-5})	Surviving Fraction	Induced Mutation Frequency (x 10^{-5}
Bastrop Soil				
10	0.90	0.55	1.6	<0
18.75	0.80	1.2	1.1	<0
25	0.71	0.29	0.99	<0
37.5	0.61	1.3	0.95	0.33
Norwood Soil				
144	0.94	<0	0.97	<0
288	0.75	0.53	0.92	0.37
566	0.69	0.74	0.99	0.40
755	0.52	2.2	1.00	<0
1133	0.41	2.8	0.97	<0

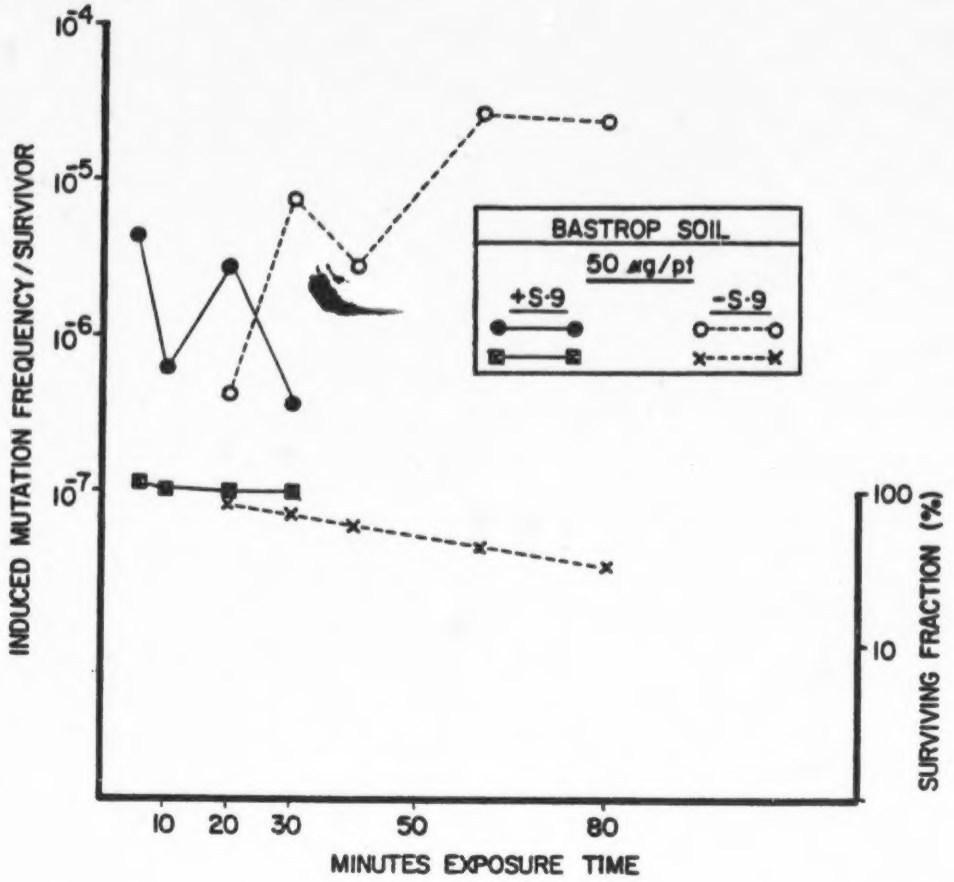


Figure 50. Induced mutation frequency and fractional survival in *A. nidulans* following exposure to organic extract of Bastrop soil. Data based on results from two independent experiments.

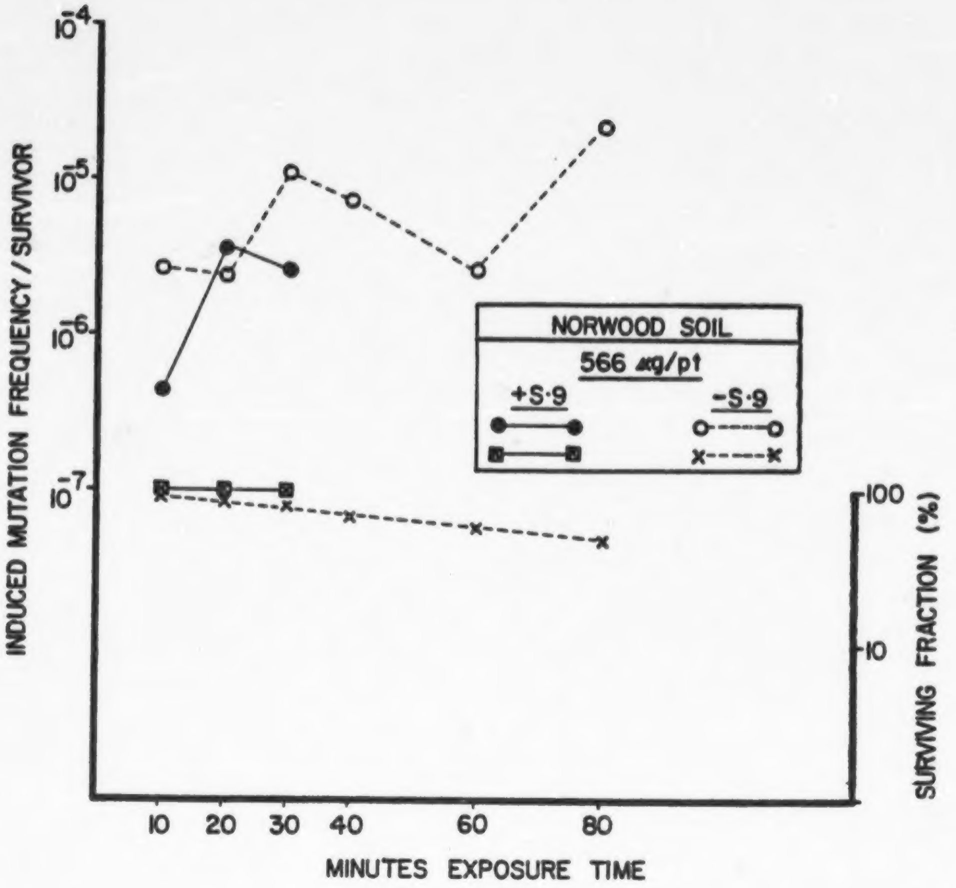


Figure 51. Induced mutation frequency and fractional survival in *A. nidulans* following exposure to organic extract of Norwood soil. Data based on results from two independent experiments.

TABLE 45. LIST OF COMPOUNDS IDENTIFIED IN NORWOOD AND BASTROP SOILS

Soil	Compound	Formula	Genetic ACtivity[1]
Norwood	Tetadecane	$CH_3(CH_2)_{12}CH_3$	P;CC
	Pentadecane	$CH_3(CH_2)_{13}CH_3$	X
	Methyl hexadecane	$C_{17}H_{36}$	X
	Hexadecane	$CH_3(CH_2)_{14}CH_3$	I
	Dimethyl hexadecane	$C_{18}H_{38}$	X
	Heptadecane	$CH_3(CH_2)_{15}CH_3$	X
	Trimethyl hexadecane		
	No octadecane	$CH_3(CH_2)_{16}CH_3$	CC
	Tetramethyl hexadecane	$C_{20}H_{42}$	X
	Nonadecane	$CH_3(CH_2)_{17}CH_3$	X
	Eicosane	$CH_3(CH_2)_{18}CH_3$	P;CC
	Heneicosane	$CH_3(CH_2)_{19}CH_3$	X
	Docosane	$CH_3(CH_2)_{20}CH_3$	X

TABLE 45 CONTINUED.

Soil	Compound	Formula	Genetic Activity[1]
Bastrop	Dodecanol	$C_{12}H_{26}O$	P;CC
	Ethyl methyl pentanol	$C_8H_{18}O$	
	Dimethyl undecane	$C_{13}H_{28}$	X
	Trimethyl decane		X
	Octadecane	$C_{18}H_{38}$	CC
	Trimethyl dodecane	$C_{19}H_{40}$	X
	Nonadecane		
	Benzenedicarboxylic acid	$C_8H_{16}O_4$	MO;CO
	Eicosane	$C_{20}H_{42}$	P;CC
	Heneicosane		
	Docosane	$C_{22}H_{46}$	C

1. Probable genetic activity: P = promotor; I = inhibitor; CC = cocarcinogenic X = unknown; References listed in test.

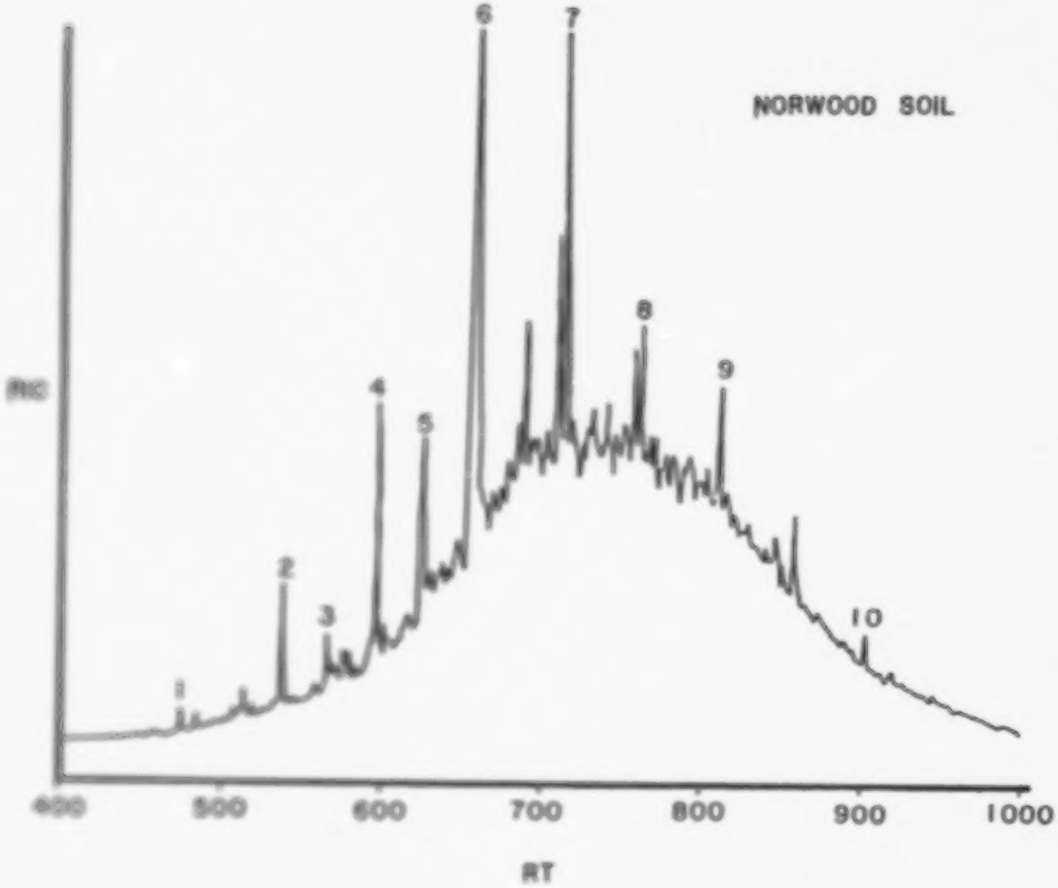


Figure 52. GC/MS chromatograph of organic extract of Norwood soil.

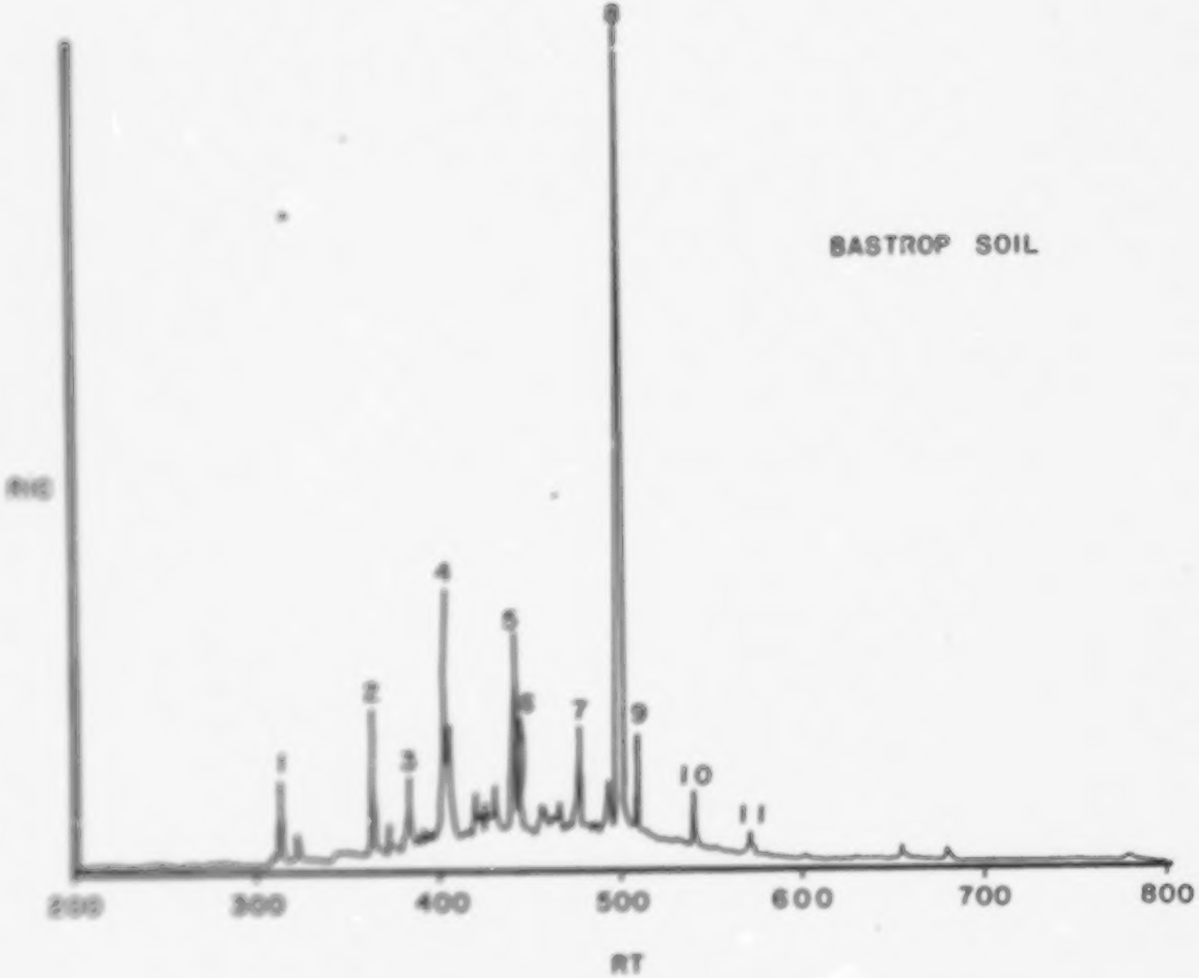


Figure 53. GC/MS chromatograph of organic extract of Bastrop soil.

soil include two alcohols, dodecanol and ethyl methyl pentanol, and benzene dicarboxylic acid. The conclusive determination of the source of the compounds identified in the soil extracts is difficult given the limited amount of available information. A chemical characterization of the resin- and asphalt-free components from a mineral soil by Morrison and Bick (1967) identified a number of n-alkanes, n-alkan-2-ones, and primary n-alkanols. The n-alkanes comprised approximately 6.4% of the wax fraction of the soil and consisted of a mixture of n-alkanes with carbon numbers ranging from 16 to 31 (Morrison and Bick, 1967). In addition, Ogner and Schnitzer (1970a) identified normal plus branched-cyclic alkanes in the water-soluble soil fulvic acid. An alternative source of the compounds identified in the Norwood and Bastrop soil is the particulate matter from diesel tractors used to cultivate the soil. One of the major organic constituents identified in diesel particulate matter is n-tetradecane (Yergey _et_ _al_., 1982). Although this and other alkanes have been identified in particulate matter, the absence of the more complex polycyclic aromatic hydrocarbons indicates that the source of the alkanes in the soil extracts is probably not diesel particulate.

The Bastrop soil also contained several alkanes in addition to two alcohols and benzene dicarboxylic acid. The most probable source of the alkanes is the waxy fraction of the soil (Morrison and Bick, 1967), whereas the alcohols may be hydroxylated metabolites of soil alkanes. The initial oxidative reaction in alkane decomposition generally involves one terminal methyl group and results in the formation of the corresponding alcohol, aldehyde, and fatty acid (Rowell, 1977). Esters of benzene dicarboxylic acid have been identified in soil organic matter (Cifrulak, 1969) and in the fulvic acid fraction of soil (Ogner and Schnitzer, 1970). Ogner and Schnitzer (1970) state that the origin of these compounds is uncertain although they may have been produced biosynthetically. One potential source of the benzenedicarboxylic acid in the Bastrop soil is the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). The primary pathway for 2,4-D biodegradation is through dichlorocatechol with complete dehalogenation usually following cleavage of the benzene ring (Bourquin and Gibson, 1978). It is possible, however, that dehalogenation of the 2,4-D in the Bastrop soil preceded ring cleavage, resulting in the formation of benzenedicarboxylic acid. Benzenedicarboxylic acid may also have reached the Bastrop soil as a result of its use as a plasticizer, pesticide carrier (Fishbein, 1979), or as a trace contaminant of the pesticides (EPA, 1980).

The results from the biological analysis of the organic extract of these soils indicate that mutagenic activity may be associated with the waxy fraction (alkanes). Mutagenic activity was detected in all three soils, with the greatest amount of activity detected in the Bastrop soil. The extract of the Bastrop and Norwood soils induced increased mutation frequencies in both the _Salmonella_ and _Aspergillus_ assays, although the mutagenic activity in _Aspergillus_ was significantly reduced in the presence of metabolic activation. The discrepancies between the results of the bioassays may have resulted from several factors. While the sensitivity of the *Aspergillus* bioassay is reported to be 96% (Scott *et al.*, 1982), there are some carcinogens which give a positive response in *Salmonella* and no response in *Aspergillus* (Bignami *et al.*, 1980). It is also possible that *Aspergillus*, which is more metabolically competent than *Salmonella* (Dunkel, 1981), was able to detoxify mutagenic compounds with the aid of microsomal activation.

Using chemical analysis, only saturated n-alkanes were detected in the extract of the Norwood soil. Of the identified compounds n-tetradecane has been found to have promoting activity (Lankas *et. al.*, 1978) and cocarcinogenic activity with benzo(a)pyrene (Goldschmidt, 1981); whereas, octadecane and eicosane were also found to have cocarcinogenic activity, and hexadecane acted as an inhibitor of benzo(a)pyrene (Goldschmidt, 1981). The mutagenic activity in the Bastrop soil may have been produced by degradation products from previously applied herbicides. Benzene dicarboxylic acid, one of the constituents of the Bastrop soil, is non-mutagenic in the *Salmonella* assay (Omori, 1976). In addition, while 2,4-D, 2,4,5-T, and mixtures of these compounds gave negative results in the *Salmonella* assay (Anderson *et al.*, 1972), a review of the literature by Grant (1979) indicates that component(s) of 2,4,5-T can cause various types of chromosome damage. Interactions of the metabolites of biodegradation represent another possible source of the mutagenic activity in soil extracts. Catechol, a frequent metabolite from the oxidation of aromatic hydrocarbons in soil (Gibson, 1971), has been found to enhance the mutagenicity of polycyclic aromatic hydrocarbons (Yoshida and Fukuhara, 1983). Other soil metabolites including dodecanol, octadecanol, and eicosaine have been found to have cocarcinogenic activity (Goldschmidt, 1981).

The biological analysis of the organic extract of three soils used solely for agricultural purposes has demonstrated the presence of mutagens and potential carcinogens. These results are in agreement with those of previous reseachers who have demonstrated that agricultural soil (Goggelman and Spitzauer, 1982; Withrow, 1982) and municipal water obtained from an agricultural area (Heartlein *et al.*, 1981) may contain significant quantities of mutagenic activity. In addition, epidemiologic studies have identified a higher incidence of certain types of cancer in the residents of rural communities (Lilienfeld *et al.*, 1972; Higginson, 1980) which may be a result of the use of agricultural chemicals. A chemical analysis of two of the three soils used in this research was unable to conclusively identify the mutagenic contaminants. However, the past history of these soils indicates that the most probable source of mutagenic activity is trace quantities of the partially oxidized residues from previous biocide applications. It also appears that the mutagenic activity of these trace contaminants may have been enhanced by the presence of promotors and cocarcinogens.

This research has defined the level of background contamination in three soils. While chemical analysis was unable to identify any mutagenic compounds, biological analysis indicated that the soil that had received the most recent applications of herbicide yielded the greatest amount of mutagenic activity. Natural sources cannot be ruled out as being responsible for the elevated mutagenic activity of the Bastrop soil; however, the presence of direct-acting mutagens in the soil indicates an exogenous source, since natural mutagens such as mycotoxins and plant toxins are indirect acting (Garner _et_ _al_., 1982; Brown and Dietrich, 1979). The presence of organic mutagens in the soil does not present a human health risk provided these materials are retained in the soil. Indeed, organic mutagens in soil may enhance the capacity of soil microorganisms to degrade complex molecules (Poglazova _et_ _al_., 1967). These results have also demonstrated the utility of a combined testing protocol. Biological analysis defined the level of mutagenic activity in each soil, while chemical analysis indicated that the activity was not produced by an artifact, e.g., histidine or solvent residues, from the collection or extraction process.

SECTION 7

QUANTIFYING SOIL EXTRACTION PROCEDURES

INTRODUCTION

Industrial activities generate large quantities of hazardous waste that must be disposed of in an economical and environmentally sound manner. These wastes have been shown to contain constituents that may be mutagenic, carcinogenic, or teratogenic. Chronic exposure of large populations to genotoxic compounds in hazardous wastes represents a serous threat to public health.

Genotoxic compounds constitute a relatively small group of agents which are highly specific for nucleic acids and produce deleterious effects in genetic elements at subtoxic concentrations (Brusick, 1980). An environmental mutagen is an agent that is released into the environment and can alter the genome or the proper functioning of a genome (Plewa, 1981). Environmental mutagens have the potential of changing human genetic material by inducing mutations. Mutations affecting somatic cells may induce cancer, heart disease, aging, or other illnesses (Ames, 1979), whereas mutations affecting germ cells may induce birth defects, sterility, or other teratogenic effects (Brusick, 1980). A carcinogen is an agent that significantly increases the yield of malignant neoplasms in a population (Clayson, 1962). A teratogen is an agent that acts during pregnancy to produce physical or functional defects in the embryo, fetus, or offspring (Meyers and Beyler, 1981). Evidence that most carcinogens are mutagens lends validity to the theory that cancer can be caused by somatic muations or damage to DNA (Brusick, 1980).

Hazardous waste must be disposed of properly in order to prevent environmental contamination and the transmission of genotoxic effects to future generations. The usual methods for disposal have been incineration, landfilling, and deep well injection. Because these techniques of waste disposal are not economical and/or do not provide for the desired destruction of genotoxic constituents, their utility is limited. Using non-destructive methods of waste disposal on the land results in sites that must be permanently removed from productivity. However, land treatment of a waste can result in land that may be reclaimed and may provide for the destruction of the genotoxic organic waste constituents (Donnelly and Brown, 1981). When properly designed and managed, a land treatment facility provides for the recycling of nutrients and water, while toxic organic constituents are retained and degraded in the upper layer of soil or soil treatment zone (Brown *et al.*, 1982a). In order to prevent contamination of the environment with mutagenic compounds, land application of hazardous waste is restricted by Environmental Protection Agency (1982) regulations to include only those wastes in which the hazardous constituents can be completely degraded, transformed, or immobilized in the soil treatment zone.

The present research is an integral part of a larger project to evaluate land treatment as an environmentally sound alternative in hazardous waste disposal. This research was designed to evaluate the efficiency of extraction procedures for recovering mutagenic activity from soil using the *Salmonella*/microsome assay (Ames *et al.*, 1975) and high performance liquid chromatography (HPLC) analysis. Microbial test systems such as the *Salmonella*/microsome assay can be used to evaluate the potential of hazardous waste constituents for causing genetic damage and, also, to accurately predict the fate and mobility of mutagenic constituents when hazardous wastes are land applied (Donnelly and Brown, 1981).

Hazardous industrial wastes contain a complex mixture of chemicals which may have additive, synergistic, or antagonistic interactions with regard to their toxic or genotoxic effects. These interactions are further complicated when wastes are mixed with the soil. Chemical analysis alone may fail to account for the transformations in the soil or various interactions which occur between chemicals in a complex mixture (Donnelly and Brown, 1981). The *Salmonella*/microsome assay provides a rapid and inexpensive means of measuring the mutagenic potential of chemicals in a soil extract.

This segment of the research evaluated two extraction procedures and determined which method most efficiently extracted the mutagenic activity from the soil. An efficient extraction procedure is a critical element for monitoring a land treatment facility. Levels of mutagenic activity at a chosen site must be monitored in order to evaluate the degradation rate of toxic organic chemicals.

MATERIALS AND METHODS

The procedures for this study involved the selection and treatment of the soils and the organic compounds. A comparison of soil extraction procedures and their reproducibility on treated soils was evaluated using the *Salmonella*/microsome assay and HPLC analysis. Statistical analysis was by the ANOVA procedure and the Duncan Multiple Range test (Steel and Torrie, 1976; Freund, 1982).

Selection of Mutagenic Compounds and Soils

Most of the experiments involved the use of either 2-nitrofluorene (Aldrich, Milwaukee, Wis) or benzo[a]pyrene (Sigma, St. Louis, Mo.). In addition, a complex waste mixture of known mutagenic activity was also used for comparison. The *Salmonella*/microsome assay is sensitive to 2-nitrofluorene (2NF), benzo[a]pyrene (B[a]P) (McCann and Ames, 1977), and the complex waste mixture, i.e., a wood-preserving bottom sediment (Donnelly *et al*., 1982). B[a]P and 2NF were selected because they are nonpolar and polar compounds, respectively; therefore, they may have different binding affinities to the soil. This may effect the efficiency of the extraction procedures to recover the cmpounds.

Two soils including a Norwood sandy clay (Typic Udifluvent) and a Bastrop sandy clay loam (Udic Paleustalf) were used. The soil characteristics are given in Table 42.

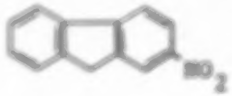
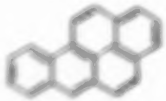
Analysis of specific organic compounds in the soils that may influence the mutagenicity of chemical additions to the soil were carried out by the U.S. Environmental Protection Agency Robert S. Kerr Environmental Research Laboratory. Research on the mutagenic activity of untreated soil is necessary because information in the literature on this topic is almost nonexistent. Chemical constituents found in the soil may enhance or reduce mutagenic activity or may effect the efficiency of extraction. A comparison was made between the mutagenic response of the known mutagens, the soil extract, and the known mutagens or waste after soil incorporation to determine the effect of soil incorporation on the mutagenicity of these mixtures.

Soil Preparation and Chemical Addition

Each soil was sieved through a 4 mm sieve and oven dried at 100°C for 24 hours. To prevent biological degradation from influencing the test results, the soils were autoclaved before chemical addition. The soil was measured into glass jars and heat sterilized for 90 min at 121°C and 15 psi for three consecutive days. The soils were brought to field capacity of 18 and 22% for the Norwood and Bastrop soils, respectively, with sterile deionized water. All soils were checked for sterility by plating on nutrient agar plates before chemical addition.

The chemicals that were used were of the highest purity available. A description of the chemicals used in this segment of the research is provided in Table 46. Treatment levels were selected to fall within the detection limits of the *Salmonella*/microsome test. Soils were treated with three different dose levels of the chemical; the dose levels were selected from the optimum response in the *Salmonella*/microsome assay. The dose at which the maximum mutational response occurred was the medium dose level. The high and low dose levels were an order of magnitude higher and lower than the optimum dose level for B[a]P ([illegible] ug/plate). For 2NF, the high dose level was half an order of magnitude higher than

TABLE 16. DIAGNOSTIC MUTAGENS USED TO EVALUATE THE EFFICIENCY OF EXTRACTION PROCEDURES

Compound	Structure	Mutagenicity	Polarity	Source
2-Nitrofluorene		Direct Acting	Polar	Aldrich, Milwaukee, WI
Benzo[a]pyrene		Indirect Acting	Nonpolar	Sigma, St. Louis, MO

the optimum dose, and the low dose level was an order of magnitude lower than the optimum dose (25 μg/plate). Soils were spiked with the chemicals or wood-preserving waste and incubated in a closed environment at room temperature for different time intervals to be studied prior to extraction.

Extraction Procedure and Sample Preparation

Hydrocarbons were extracted from the soil using a Waring laboratory blender following procedures of Brown and Donnelly (1983). A Soxhlet blender technique was used on the wood-preserving bottom sediment to compare the efficiency of both extraction techniques (Brown *et al.*, 1982b). The extracting solvent, dichloromethane, was removed by evaporating the sample to near dryness on a Brinkman-Bucci Rotary Evaporator. Dichloromethane was used to transfer the organic residue to a glass culture tube. The sample was taken to dryness under a stream of nitrogen. The dried extract was dissolved in 5 ml dimethyl sulfoxide (Sigma, St. Louis, Mo.) and brought to its original concentration with reference to the low, medium, or high dose level. The samples were filtered through a 0.45 μm millipore filter (Millipore Corp., Bedford, Mass.) to remove soil particles. The sample was serially diluted in dimethyl sulfoxide to a 10^{-3} dose level so that four concentrations of each soil dose level were tested in the mutagenicity assay.

Bioassay

The *Salmonella*/microsome mutagenicity test was used to measure the mutagenic potential of the extracted samples. Dilutions of each extract were tested with *Salmonella typhimurium* strain TA98 supplied by Dr. B. N. Ames, University of California at Berkeley, CA. *Salmonella* TA98 is a frameshift mutant which contains a histidine D3052 mutation and a deletion which invokes a requirement for biotin. Frameshift mutations occur by shifted pairing in repetitive sequences of DNA. The mutation is in a reverse direction to independence from histidine auxotrophy. This bacteria strain is capable of being reverted from a histidine requirement back to prototrophy by a wide variety of mutagens.

Overnight bacterial cultures were grown in Oxoid Nutrient Broth No. 2 (KC Biological, Lenexa, Kansas) with shaking at 37°C for 16 hours. Liver homogenates (S9 from rats induced with a polychlorinated biphenyl mixture [Aroclor 1254]) (Litton Bionetics, Charleston, S.C.) were incorporated into the test system to allow indirect acting mutagens to reach their ultimate mutagenic form. This incorporates an important aspect of mammalian metabolism into the test, thereby allowing those carcinogens requiring metabolic activation to be as easily detected as mutagens. The procedures used for the preparation of the S9 mix are described by Ames *et al.* (1975). Positive, solvent, and sterility controls were included in each test to act as an internal control for the bioassay, to demonstrate the sensitivity of the test system, and to demonstrate the functioning of the metabolic activation system.

High Performance Liquid Chromatography

Samples were assayed using HPLC to quantify the recovery of the chemicals from soil. Techniques used for HPLC are as described by Schram (1980) and Yost *et al*. (1980). The analysis of polynuclear aromatic hydrocarbons utilizes octadecylsilane bonded stationary phases and acetonitrile/water as the mobile phase (Belinki, 1980). To optimize the mobile phase composition, an isocratic composition of acetonitrile/water was used. This compound is favored as it permits faster separations than other solvent systems (Donahue *et al*., 1978). Acetonitrile has a much lower viscosity than does methanol; therefore, a faster flow rate can be used without exceeding the pressure limit of the column. Also, acetonitrile has a greater ability to dissolve many polycyclic hydrocarbons than other solvents, thus greater amounts can be loaded into the system per injection (Donahue *et al*., 1978).

RESULTS AND DISCUSSION

In order to determine the affect of storage on the extraction of mutagenic activity from soil, the polar compound 2-nitrofluorene or the nonpolar benzo(a)pyrene was added to the two soils and extracted following 24 hours or 7 days of storage at 0°C. The results from biological analysis of the extract of the Norwood or Bastrop soils amended with the two compounds indicated that there was no statistical difference ($p<.01$) between samples stored for 24 hours or 7 days. These results demonstrate that binding of chemicals to soil particles will not affect the mutagenic activity of sterile soil stored at 0°C for at least one week.

The results from the biological analysis of the extract of the two soils amended with 2-nitrofluorene are provided in Table 47 and Figures 54 and 55. At the optimum dose level (25 µg), 2-nitrofluorene induced 85 revertants/µg, while the extract from 500 mg of the Norwood and Bastrop soils induced .13 and .11 revertants/mg, respectively. At the highest treatment level (25 mg 2NF/50 g soil), the 2-nitrofluorene and 2-NF amended Norwood and Bastrop soils induced 5.4, 6.1, and 5.2 revertants/mg, respectively. All of these values fall within their respective standard deviations. Both the 10^{-1} and 10^{-2} dilutions at the high treatment level for the two soils fell slightly below the anticipated level for 2-nitrofluorene. At the medium and low treatment levels, the mutagenic activity of the extract of the 2-NF amended Bastrop and Norwood soil was within the standard deviation for the pure chemical at all dose levels. These results indicate that a blender extraction of soil amended with the direct-acting polar compound 2-nitrofluorene will provide complete recovery of the mutagenic activity in the soil.

TABLE 47. MUTAGENIC ACTIVITY, AS MEASURED WITH S. TYPHIMURIUM TA98, OF 2-NITROFLUORENE, AND THE SOLVENT EXTRACT OF THE NORWOOD AND BASTROP SOILS AMENDED WITH VARIOUS LEVELS OF 2-NITROFLUORENE

Sample	Dose/Plate (mg)	2-Nitrofluorene	Bastrop Soil	Norwood Soil
None	0	-	55 ± 18	64 ± 31
	-1	-	39 ± 17	31 ± 12
	-2	-	34 ± 10	26 ± 10
	-3	-	32 ± 11	25 ± 10
2-nitrofluorene (high)	0(0.5)	2711 ± 478	3074 ± 509	2600 ± 202
	-1(0.05)	-	2632 ± 373	2484 ± 226
	-2(0.005)	-	1145 ± 251	966 ± 113
	-3(0.0005)	-	239 ± 55	152 ± 20
(med)	0(0.25	2560 ± 409	2490 ± 271	2574 ± 518
	-1(0.025)	2122 ± 465	1870 ± 177	2231 ± 289
	-2(0.0025)	682 ± 173	490 ± 80	593 ± 66
	-3(0.00025)	123 ± 30	93 ± 24	103 ± 12
(low)	0(0.025)	2122 ± 465	2056 ± 183	1930 ± 535
	-1(0.0025)	682 ± 173	549 ± 47	825 ± 331
	-2(0.00025)	123 ± 30	117 ± 16	153 ± 46
	-3(0.000025)	30 ± 7	31 ± 4	36 ± 13

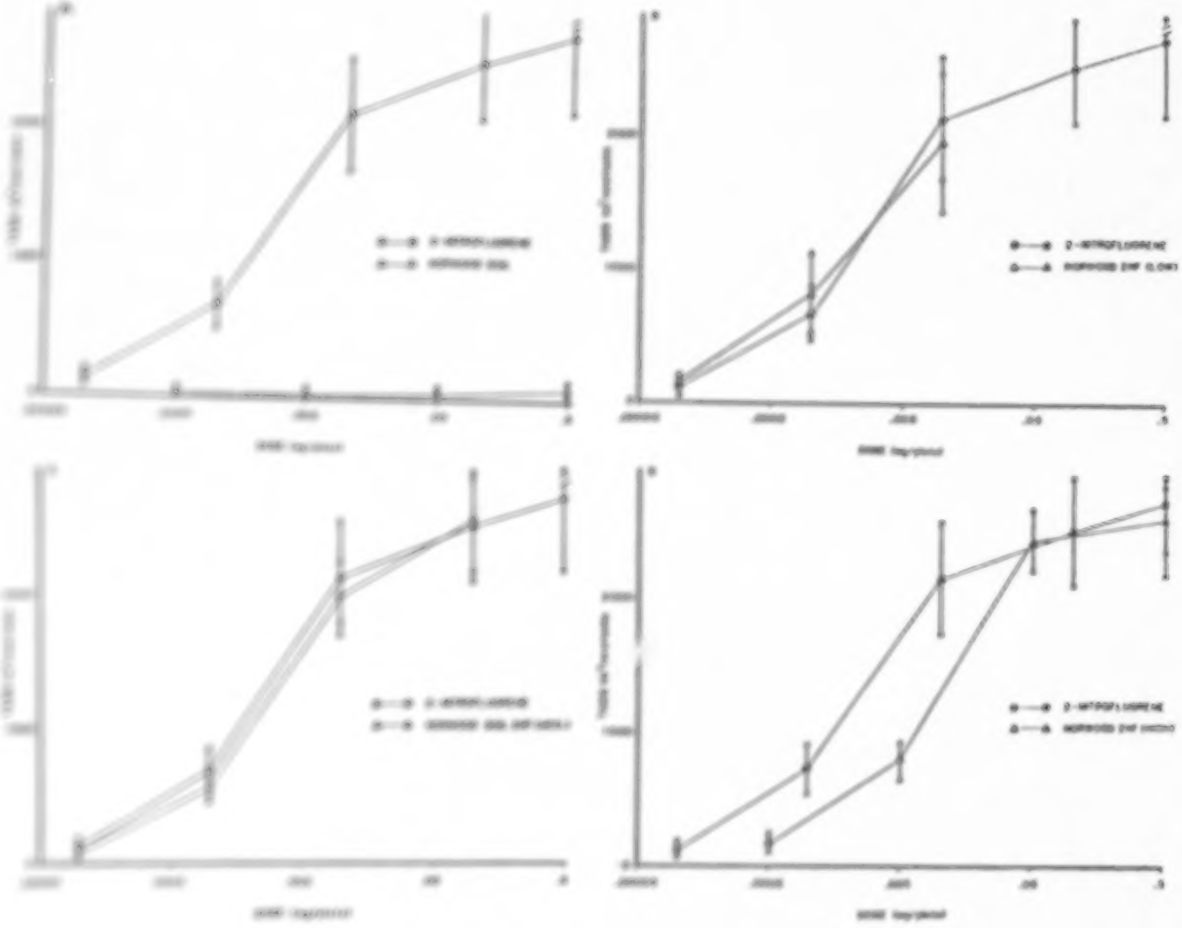


Figure 56. Extraction efficiency, as measured with S. typhimurium strain TA98, of 2-nitroflourene from Norwood soil.

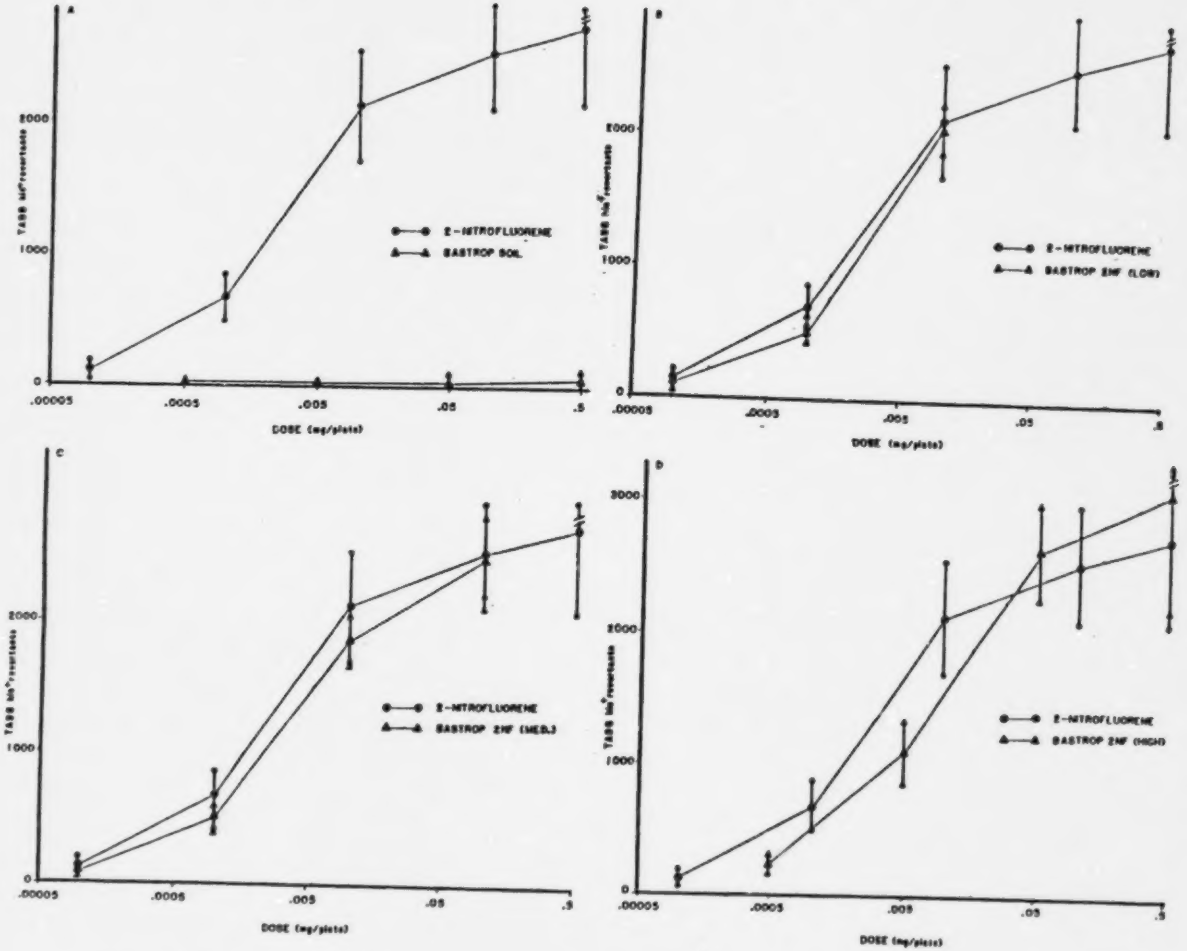


Figure 55. Extraction efficiency, as measured with S. typhimurium strain TA98, of 2-nitrofluorene from Bastrop soil.

Additional tests were conducted using the nonpolar indirect-acting benzo(a)pyrene. The results from biological analysis of the extracts of the two soils amended with benzo(a)pyrene are provided in Table 48 and Figures 56 and 57. For both soils at all treatment levels, these was no appreciable difference in the mutagenic activity of the B(a)p and the soil amended with B(a)p. At the high and medium treatment levels, the extract of the Norwood soil plus B(a)p was consistently less mutagenic than the pure compound. However, the soil extract did fall within the standard deviation of the pure compound at the same dose levels. Thus, there was no apparent interraction between the benzo(a)pyrene and the soil components which significantly affected the mutagenic acitivity of the soil extract.

The soil extracts were also analyzed using high performance liquid chromatography to determine the efficiency of the blender extraction procedure. The HPLC chromatograms from the solvent control, the two soils, and the soil plus the chemicals are provided in Figures 58 through 63. For the Norwood soil, the average extraction efficiency for both compounds at all three treatment levels was 91% (Table 49), while the average efficiency for the Bastrop soil was 89% (Table 49). The average extraction efficiency for benzo(a)pyrene and 2-nitrofluorene in the Norwood soil was 91 and 99%, respectively. For the Bastrop soil, the average recovery was 84% for benzo(a)pyrene and 93% for 2-nitrofluorene. These results indicate that there was no appreciable difference in the extraction efficiency from the Norwood or Bastrop soils. Treatment level did not appear to influence the recovery of benzo(a)pyrene, whereas the recovery of 2-nitrofluorene at the low treatment level was 15% less than the high and medium levels.

When the extraction efficiencies are compared for each of the pure compounds, the recovery of the polar compound appears to be slightly greater. The extraction efficiency from both soils at all three treatment levels was 90% for the nonpolar benzo(a)pyrene and 96% for the polar 2-nitrofluorene. The lower efficiency observed in the benzo(a)pyrene amended soils was influenced by the very low recovery efficiency in the Norwood soil at the medium treatment level. The cause of the low recovery efficiency for benz(a)pyrene in the Norwood soil is at this point unknown.

Biological and chemical analysis of two soils amended with either 2-nitrofluorene or benzo(a)pyrene have indicated that the blender extraction procedure does provide adequate recovery of mutagenic compounds. The results from the Salmonella/microsome assay indicate that there was no appreciable difference in the mutagenic activity of the pure compound or the extract of the soil amended with the pure compound at equivalent dose levels. In addition, the extraction efficiency as measured using high performance liquid chromatography averaged greater than 85% for both chemicals at all treatment levels on both soils. This analytical approach, using combined chemical and biological analysis, has demonstrated that for the compounds, levels,

TABLE 48. MUTAGENIC ACTIVITY, AS MEASURED WITH *S. TYPHIMURIUM* TA98, WITH METABOLIC ACTIVATION, OF BENZO(a)PYRENE, AND THE SOLVENT EXTRACT OF THE NORWOOD AND BASTROP SOILS AMENDED WITH VARIOUS LEVELS OF BENZO(a)PYRENE

Sample	Dose/Plate (mg)	B[a]P	Bastrop Soil	Norwood Soil
None	0	-	64 ± 5	56 ± 23
	-1	-	47 ± 8	42 ± 22
	-2	-	44 ± 9	39 ± 19
	-3	-	45 ± 14	40 ± 21
B[a]P (high)	0(0.5)	500 ± 139	543 ± 79	361 ± 50
	-1(0.05)	514 ± 123	612 ± 122	400 ± 68
	-2(0.005)	505 ± 163	553 ± 124	390 ± 185
	-3(0.0005)	74 ± 33	90 ± 17	52 ± 11
(med)	0(0.05	514 ± 123	355 ± 61	437 ± 944
	-1(0.005)	505 ± 163	293 ± 195	459 ± 118
	-2(0.0005)	74 ± 33	54 ± 16	89 ± 41
	-3(0.00005)	35 ± 10	49 ± 9	58 ± 36
(low)	0(0.005)	505 ± 163	618 ± 82	475 ± 163
	-1(0.0005)	74 ± 33	119 ± 18	118 ± 71
	-2(0.00005)	35 ± 10	60 ± 11	46 ± 15
	-3(0.000005)	-	48 ± 7	43 ± 16

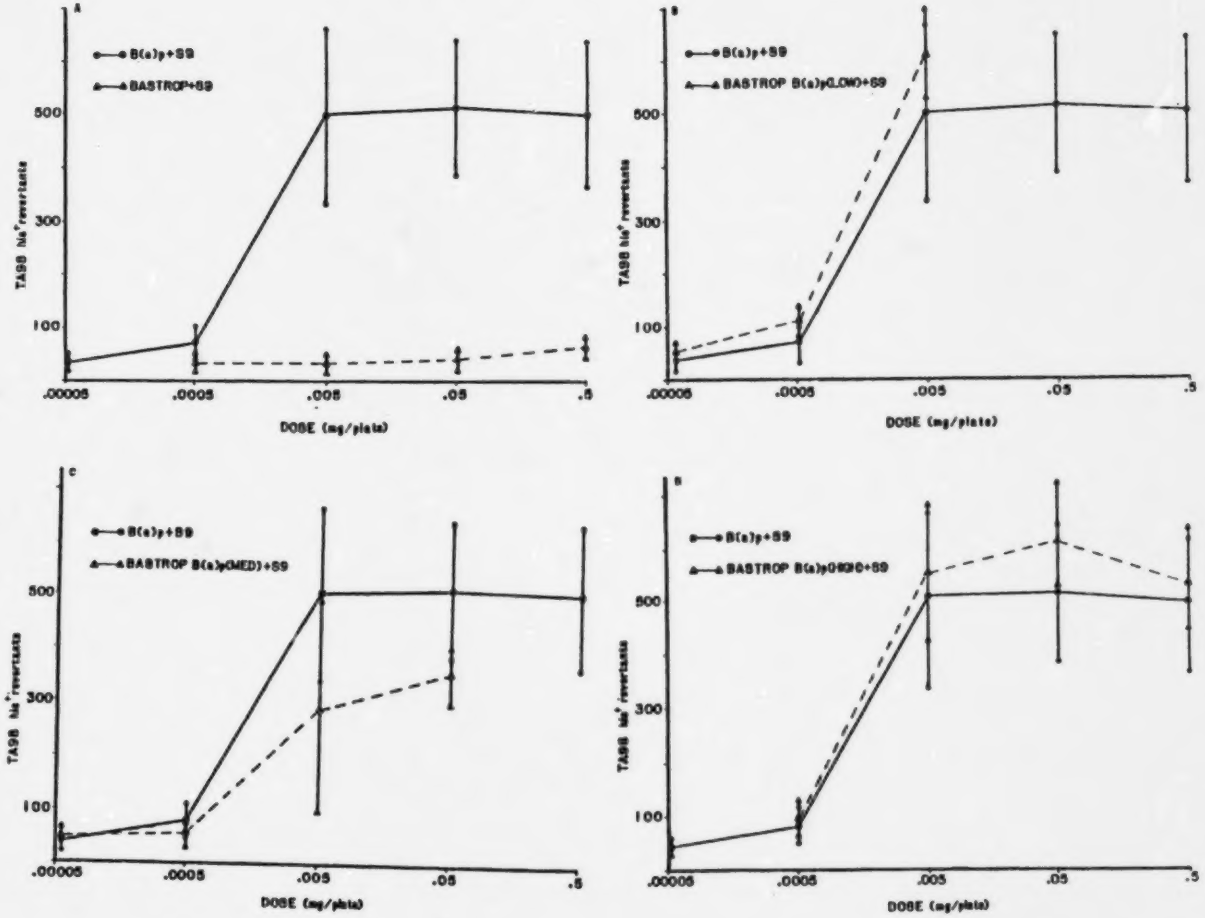


Figure 56. Extraction efficiency, as measured with S. typhimurium strain TA98, of benzo(a)pyrene from Norwood soil.

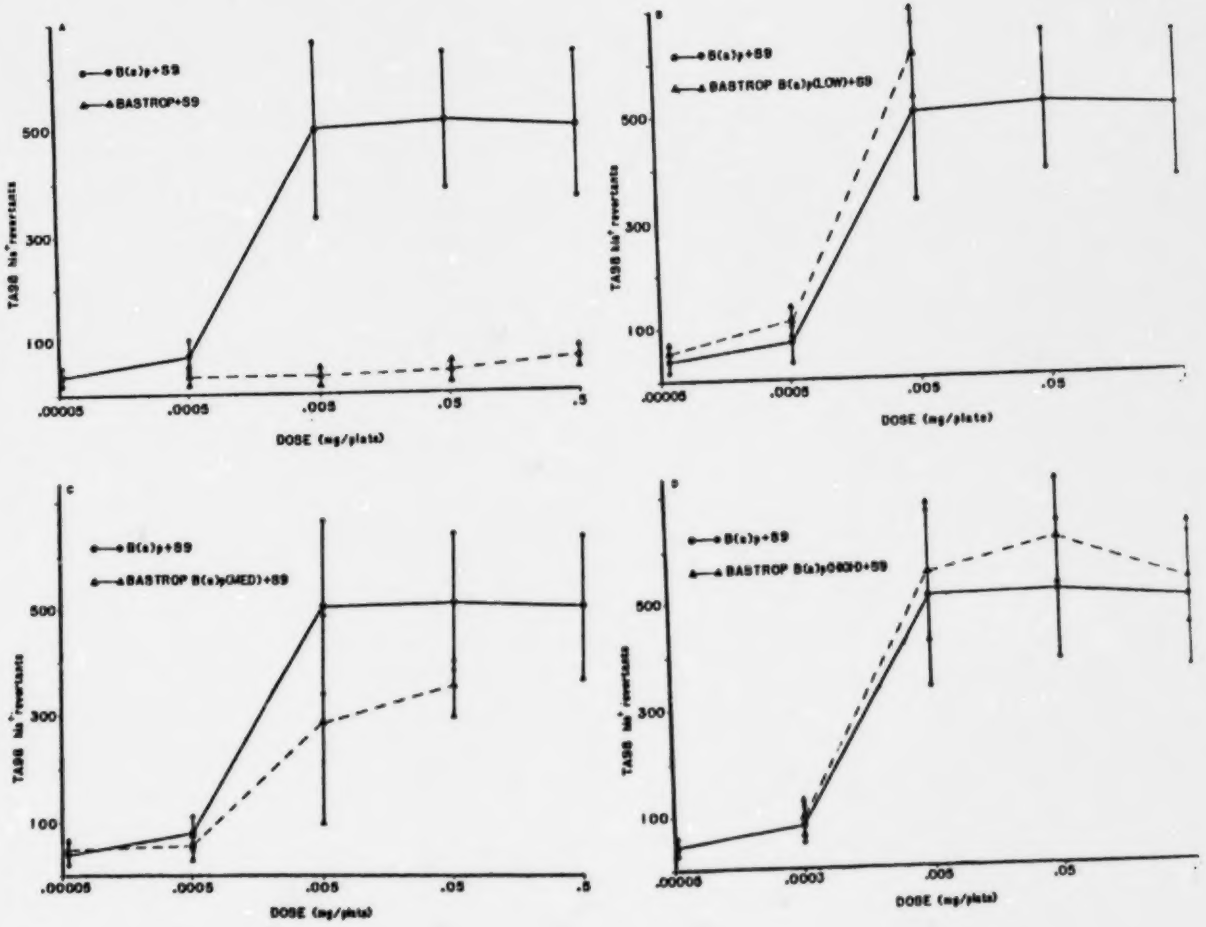


Figure 57. Extraction efficiency, as measured with S. *typhimurium* strain TA98, of benzo(a)pyrene from Bastrop soil.

TABLE 49. EXTRACTION EFFICIENCY, AS MEASURED WITH HPLC, OF THE BASTROP AND NORWOOD SOILS AMENDED WITH MUTAGENIC COMPOUNDS

Compound	Application Rate	Bastrop Soil	Norwood Soil	Average
2-Nitrofluorene	25 mg 2NF/50g soil	104	104	104
	12.5 mg/50g	91	108	100
	1.25 mg/50g	84	85	85
	Average	93	99	96
Benzo(a)pyrene	25 mg B(a)p/50g soil	89	97	93
	2.5 mg/50g	89	52	71
	.25 mg/50g	73	99	86
	Average	84	83	84
Average both compounds		89	91	90

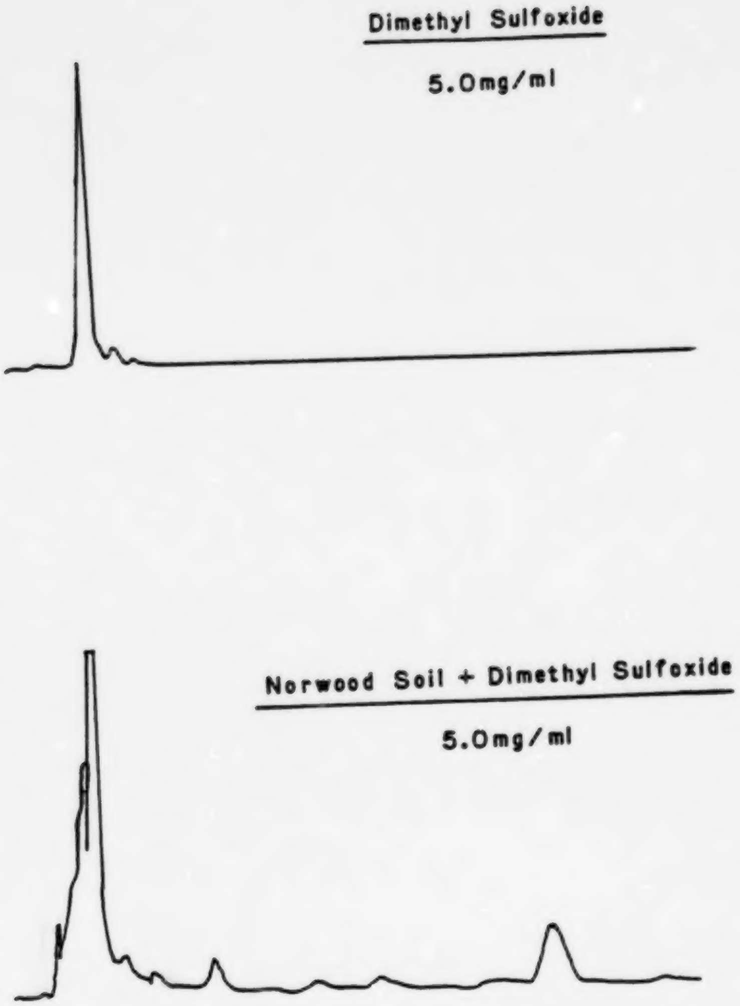


Figure 58. HPLC chromatograph from (A) dimethylsulfoxide and (B) Norwood soil.

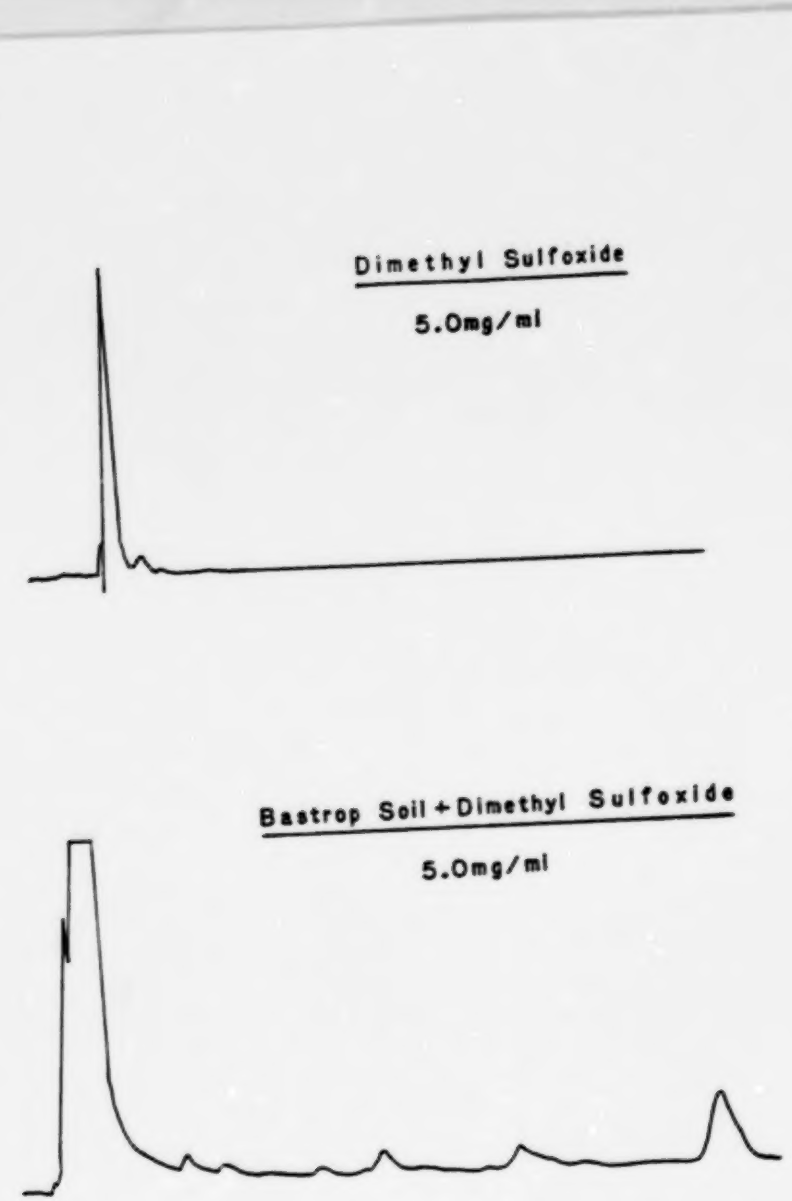


Figure 59. HPLC chromatograph from (A) dimethylsulfoxide and (B) Bastrop soil.

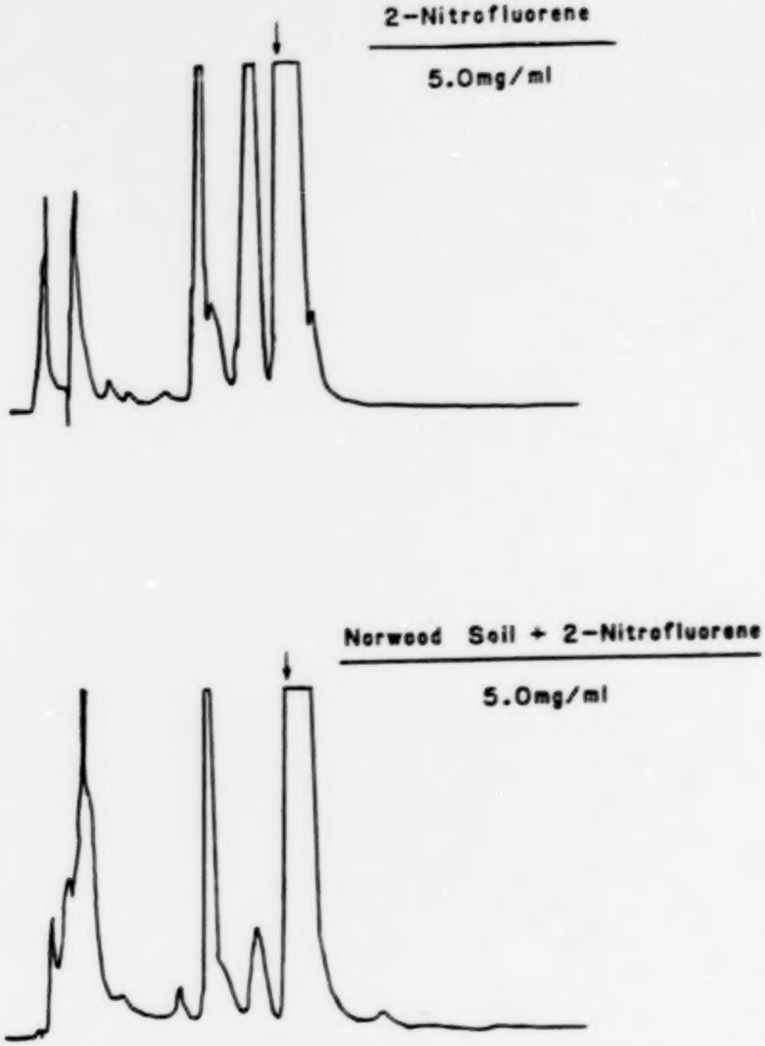


Figure 60. HPLC chromatograph from (A) 5.0 mg/ml 2-nitroflourene, and (B) Norwood soil + 5.0 mg/ml 2-nitroflourene.

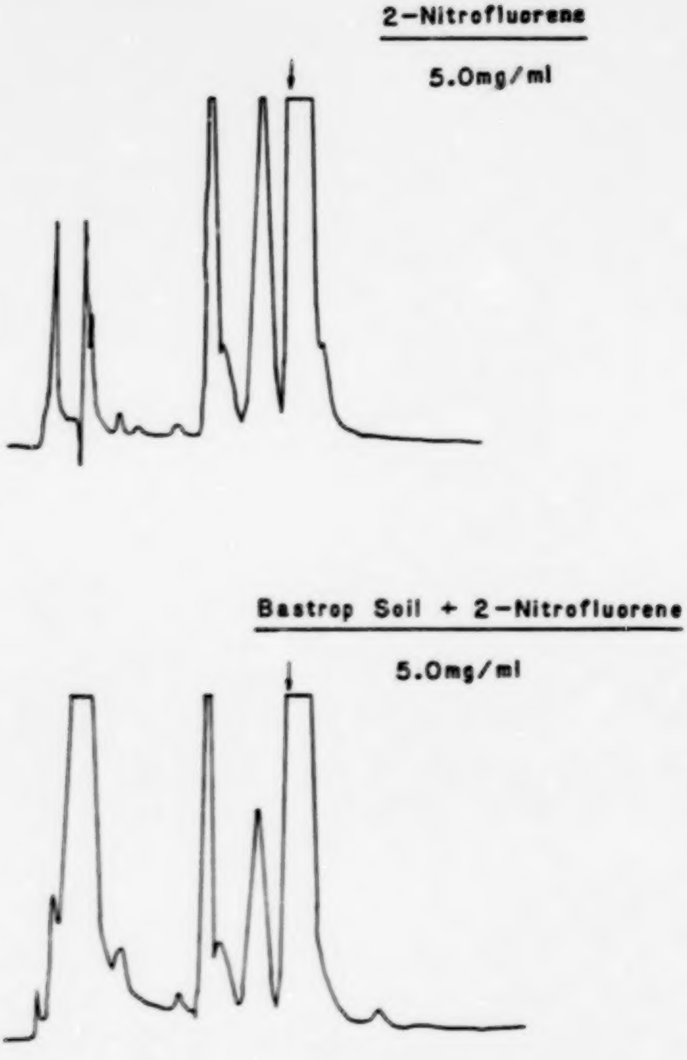


Figure 61. HPLC chromatograph from (A) 5.0 mg /ml 2-nitroflourene, and (B) Bastrop soil + 5.0 mg/ml 2-nitrofluorene.

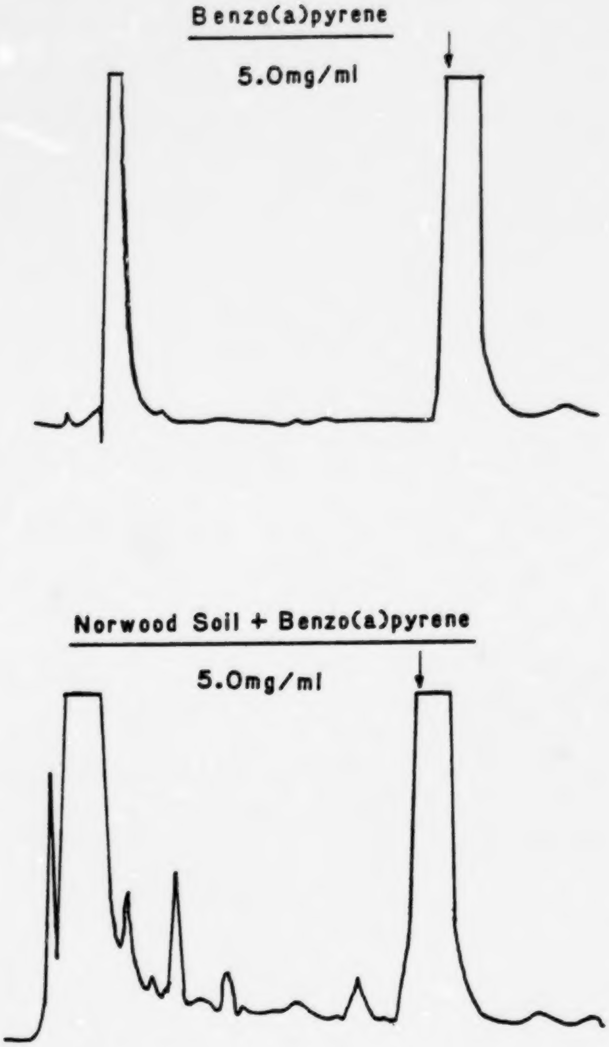


Figure 62. HPLC chromatograph from (A) 5.0 mg/ml benzo(a)pyrene, and (B) Norwood soil + 5.0 mg/ml benzo(a)pyrene.

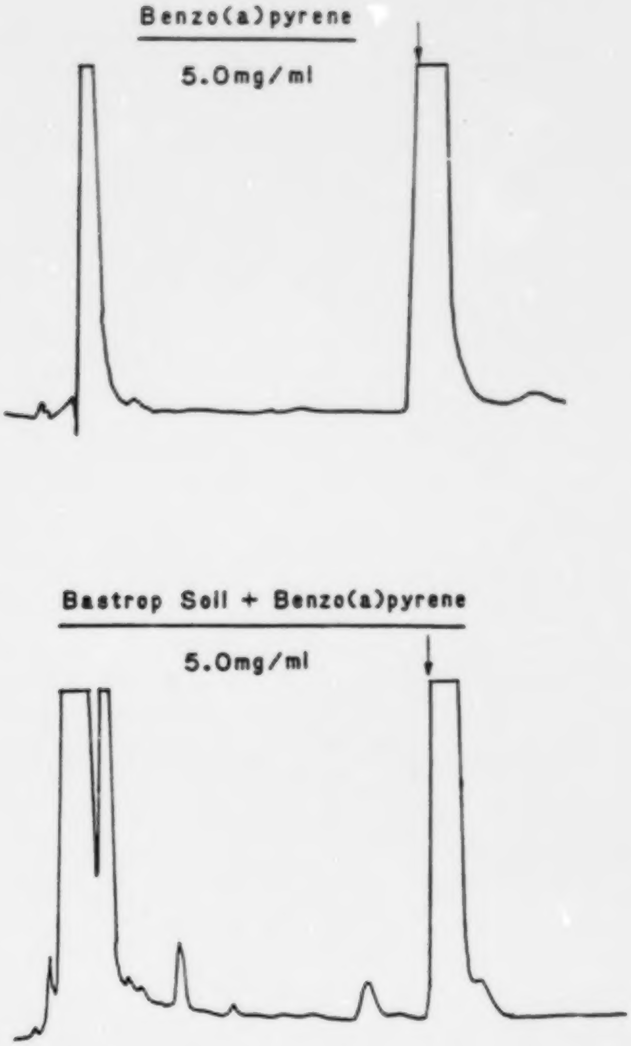


Figure 63. HPLC chromatograph from (A) 5.0 mg/ml benzo(a)pyrene, and (B) Bastrop soil + 5.0 mg/ml benzo(a)pyrene.

and soils evaluated, there are no interractions with soil compounds, and the blender procedure does provide efficient extraction of mutagenic compounds from soil.

SECTION 8

MUTAGENIC ACTIVITY OF RUNOFF WATER FROM HAZARDOUS WASTE AMENDED SOIL

INTRODUCTION

Land treatment of hazardous waste consists of a program of controlled applications of waste to the surface layer of soil accompanied by a carefully planned management and monitoring program. This technique is designed to protect the environment by using the surface layer of soil to transform, degrade, or immobilize hazardous waste constituents. Many of the more complex organic waste constituents, particularly those which are toxic or genotoxic, may persist in the soil for an extended period of time (Alexander, 1981). Scant information is available concerning the ultimate fate of resistant organic chemicals in the environment. These chemicals may volatilize from the soil, be translocated by plants, be removed by runoff water, or be leached into the groundwater. A variety of analytical techniques could be used for characterizing samples of the various streams leaving the soil. Detailed chemical analysis would provide information on the presence or probable presence of specific compounds. A chemical analysis would not, however, be capable of predicting the synergistic, additive, or antagonistic interactions of a mixture of chemicals or their effect on a biological system. Biological analysis provides the only analytical technique that can be used to define the toxic or genotoxic potential of an environmental sample.

This segment of the present study was conducted to evaluate the potential for the removal of mutagenic waste constituents by rainfall. The EPA (1980) regulations require that the runoff from hazardous waste land treatment facility be retained. However, monitoring techniques must be developed to determine when runoff water can be safely discharged following closure of the facility. In addition, these results will provide information on the presence of water soluble mutagens in soil and the effect of degradation on their mutagenic activity.

MATERIALS AND METHODS

Waste

Three petroleum based sludges were utilized in the greenhouse study. A general description of their characteristics is provided in

Table 50, while a more detailed description is included in Section 5. The calculated amount of waste applied to the soil in each box was based on the bulk density of each soil (1.44 g cm^{-3} for the Norwood soil and 1.49 g cm^{-3} for the Bastrop soil), the volume of soil in each box (36,225 cm^{3}), and the percent extractable hydrocarbons in each waste (27% for PENT S, 21% for SWRI, and 41% for COMBO). The weight of waste applied to each box was be determined by multiplying the weight of soil times the desired level of extractable hydrocarbons in each box and dividing this figure by the amount of extractable hydrocarbons in each waste.

Based on these calculations, the wood-preserving bottom sediment (PENT S) was applied to the greenhouse boxes at a rate of 5.5 kg/box to the Norwood soil and 6.5 kg/box to the Bastrop soil, or a hydrocarbon loading rate of 3.1%. The application rate for the storm-water runoff impoundment waste (SWRI) was 11.2 kg/box to the Norwood soil and 11.6 kg/box to the Bastrop soil, or a hydrocarbon loading rate of 4.5%. The combined API-separator/slop oil emulsion sludge (COMBO) was also applied at a hyrocarbon loading rate of 4.5%. This was equivalent to a sludge application rate of 5.7 kg/box to the Norwood soil and 5.9 kg/box to the Bastrop soil.

Soil

The physical and chemical properties of the soils used in the greenhouse study are provided in Table 42. A more detailed discussion of the Bastrop and Norwood soils is provided in Section 6. Approximately two fifty-five gallon barrels of each soil were collected from an uncontaminated site for use in the greenhouse study.

Greenhouse Boxes

At the time of collection, the soils used in the greenhouse study were slightly moist to wet. The two soils were spread separately on sheets of brown paper on greenhouse benches to air dry. Large clods were passed through an ore crusher to reduce their size. Air dried soil was passed through a 4 mm sieve and stored until needed.

Sifted soil was packed in wooden boxes (45 cm x 57 cm x 20 cm) in 5 cm lifts to a depth of 17 cm. The surface of the soil was smoothed to level with the runoff collection trough (Figure 64). Waste incorporation into the soil was accomplished by removing approximately one-half of the soil from the box and applying the waste to the remaining soil in the box. The removed soil was added to the box and mixed with a small trowel until the mixture was homogenous. Twelve boxes of each soil were prepared for the greenhouse study. These included three boxes of each soil to serve as controls and three boxes of each soil to receive the PENT S, SWRI, or COMBO waste. Soil samples were collected from greenhouse boxes before, immediately after, and 45, 90, 180, 360, and 540 days following waste application.

TABLE 50. DISTRIBUTION OF MUTAGENIC ACTIVITY[1] IN FRACTIONS OF HAZARDOUS WASTE AND THE TWO SOILS USED IN THE GREENHOUSE STUDY

Fraction		Extractable Hydrocarbon (mg/g)	Specific Activity (rev./mg)[2]	Weighted Activity (rev./mg)[3]
PENT S	Crude	270	1,282	346
	Acid	23	771	18
	Base	24	1,204	29
	Neutral	223	860	192
SWRI	Acid	8	475	4
	Base	5	418	20
	Neutral	346	420	145
COMBO	Acid	6	368	2
	Base	3	344	1
	Neutral	137	497	68
Norwood	Crude	0.057	21	0.0012
Bastrop	Crude	0.229	297	0.068

1- Mutagenic activity as measured with strain TA98 with microsomal activation.

2. Revertants/mg = slope of mutation induction curve calculated using three highest non-toxic dose levels.

3. Revertants/mg eq. = Revertants/milligram material extracted; calculated by multiplying revertants/mg x % extractable hydrocarbons.

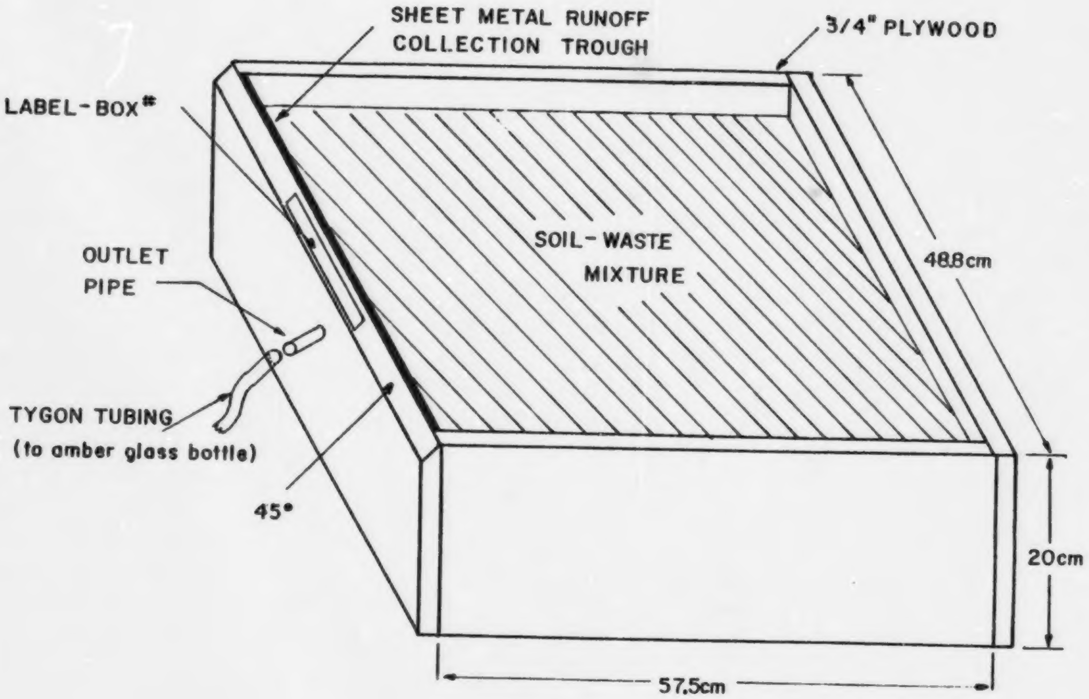

SHEET METAL RUNOFF
COLLECTION TROUGH
3/4" PLYWOOD
LABEL-BOX#
SOIL-WASTE
MIXTURE
48.8cm
OUTLET
PIPE
TYGON TUBING
(to amber glass bottle)
45°
20cm
57.5cm

Figure. 64. Schematic diagram of greenhouse boxes used in degradation study.

Runoff samples were also collected before, immediately after, and 45, 90, 180, 360, and 540 days following waste application. A rainfall simulator that held four boxes per simulation was used to generate runoff samples. The boxes were placed in the chamber on a 10° slope, and a 3.5 in/hour intensity rain was applied with a rotating disk rainfall simulator as described by Morin *et al*. (1967). The water used in the simulated rainfall was rain water collected from a trough on the sides of a fiberglass greenhouse. Collected rain water was pumped to a 600 gallon capacity storage system where it was stored until needed.

Extraction Procedures

Runoff samples were extracted using the procedures of Brown and Donnelly (1982). The samples were passed through a mixed bed of 4.0 g of XAD-2 and 6.3 g XAD-7, or approximately 20 cm^3 of each resin as suggested by Rappaport *et al*. (1979). The resins were washed prior to use by swirling and decanting three times with ten volumes each of acetone, methanol, and distilled water. Washed resins were stored at 4°C prior to use. Glass econo-columns (Bio-Rad, Richmond, CA) 1.5 x 50 cm^2 were packed with 20 cm^3 of XAD-2 resin followed by 20 cm^3 of XAD-7 resin. Dressler (1979) found that the combined XAD-2 and XAD-7 column provided the most efficient recovery for a broad range of organic compounds, while Grabow *et al*. (1981) observed that the XAD technique may be more efficient than a liquid-liquid extraction for the recovery of low concentrations of mutagens. A glass wool plug was placed above the resin in order to trap soil particles. The columns were flushed with 1,200 ml of distilled water before loading the water sample. Leachate or runoff water was placed in a reservoir and allowed to pass through the column by gravity flow at about 50 ml per minute. After loading the water sample, dry nitrogen was introduced into the column to remove the residual aqueous phase, and the column was washed with 120 ml of distilled water to remove residual histidine. The adsorbed organic compounds were eluted with 160 ml of acetone. The acetone extract was filtered through about 30 g of anhydrous Na_2SO_4 and Whatman No. 42 filter paper into a flat bottom flask. Extract were reduced to less than ten milliliters on a Brinkman-Bucci Model R roto-evaporator. The sample was added to a glass funnel filled with sodium sulfate to remove residual water and washed with acetone. The sample was then pipetted off into a small screw-capped glass culture tube and taken to dryness under a stream of nitrogen. Dimethyl sulfoxide (Sigma) was added to the dried extract, and the resultant solution was passed through a 0.2 µm average pore diameter Teflon filter (Millipore-Fluoropore Bedford, Mass.). Samples were stored at 4°C prior to use.

Chemical Analysis

The compounds were identified using a Finnigan OWA Automated GC/MS. The GC capillary column used in the procedure was a J&W Scientific

DB-5-30W (Orangeville, CA). One µl aliquots were used with a helium carrier gas flow near 36 cm/sec. The GC oven temperature program was 60°C for 1 minute and then increased at 6°/min to 260°C with a hold time of 12 minutes. The OWA unit has a splitless mode injector. The software used has a mass spectra library of 31,331 organic compounds.

Biological Analysis

The potential of the organic extracts of runoff water to induce genetic damage was measured with two microbial systems capable of detecting compounds which produce point mutations.

The *Salmonella*/microsome assay of Ames *et al*. (1975) was used to monitor the mutagenic activity of concentrated water samples. The *Salmonella* strains were kindly supplied by Dr. Bruce N. Ames (University of California, Berkeley, CA). The methods were the same as Ames *et al*. (1975) except that overnight cultures were prepared by inoculation into 10 ml of Oxoid Nutrient Broth #2 (KC Biological, Inc., Lenexa, KS) and incubated with shaking for 16 hours at 37°C. Water extracts were tested on duplicate plates in two independent experiments in the standard plate incorporation assay at a minimum of 4 dose levels of the sample with and without enzyme activation (0.3 ml rat liver/ml S-9 mix) using strains TA98 and TA100. Aroclor 1254 induced rat liver was obtained from Litton Bionetics (Charleston, SC). Positive controls included 2 µg/plate N-methyl-N'-nitro-N-nitrosoguanidine (Sigma) for TA100, 25 µg/plate 2-nitrofluorene (Aldrich) for TA98, and 10 µg/plate 2-aminoanthracene (Sigma) which was used with all strains to verify the functioning of the metabolic activation system. All reagents and extracts were tested for sterility; DMSO was used as a negative control.

The bioassay using *Aspergillus nidulans* was used to detect point mutations and small deletions induced in a haploid genome using the methionine system. Conidia from four to five single colonies of the *methG1 biA1* (requiring methionine and biotin) Glasgow strain of *Aspergillus nidulans* grown for 5 to 6 days on a complete medium at 37°C were used for each experiment. Samples were tested at one dose level, usually the extract equivalent to 250 ml of water, and one exposure time selected to yield approximately 50% of survival on five plates in two independent experiments with and without metabolic activation. The procedures used were the same as Scott *et al*. (1978) except that the cells were exposed in a 13 x 100 mm screw capped culture tube. The exposed cells were plated on a complete medium to determine survival and a methionine-free medium to determine induced mutation frequency. Mutant colonies were scored after incubation for 5 days at 37°C. Colonies were divided by colony morphology into three classes, A, B, C, as well as the total mutation frequency represented by the sum of induced mutants from Class A, B, and C. Each of these three classes is believed to involve two genes. The morphology of Class A colonies is green, Class B brown, and Class C green with a white hyaline edge. The frequency of mutations induced by a sample was determined by subtracting the frequency of spontaneous mutations from the total mutation frequency. A sample was considered mutagenic if the induced-mutation frequency was greater than 5.0. Positive controls included 8-methoxypsoralen (Sigma, St. Louis, Mo), 8-methoxypsoralen plus near UV light without activation, and benzo(a)pyrene (Aldrich, Milwaukee, WI) with metabolic activation.

RESULTS AND DISCUSSION

Runoff water was collected from the Norwood or Bastrop soil amended with PENT S waste over a 540 day period. The mutagenic activity of the residual hydrocarbons in the runoff water from the Norwood soil increased with each subsequent sampling date (Figure 65). When compared to the runoff sample from day 0, the mutagenic potential of the sample collected on day 540 was approximately eight and three times greater in strain TA98 and TA100, respectively (Tables 51 and 52). In addition, strain TA98 detected mutagenic activity without the S9 mix in the runoff sample from day 540, whereas no mutagenic activity was detected in the absence of metabolic activation in runoff samples collected prior to this date (Figure 66).

While similar results were obtained with the runoff water from the Bastrop soil, the increase in mutagenic activity over time was much less dramatic than the increase observed in the runoff water from the Norwood soil. The mutagenic potential of the residual hydrocarbons in the runoff water from the PENT S amended Bastrop soil increased from day 0 through day 360 but remained constant or decreased slightly from day 360 to day 540 (Figure 67, Tables 53 and 54). As with the Norwood soil, direct-acting mutagens were detected on day 540 (Table 53). The results from a chemical analysis of runoff water from control and PENT S waste amended Bastrop soil from day 0 and 360 are provided in Figures 68, 69 and 70. There were no compounds present in detectable levels in the runoff water from the unamended Bastrop soil (Figure 68). Ten compounds were detected in the runoff water collected on day 0 from the waste amended Bastrop soil, including dimethylnapthalene (DMN), dihydro-acenaphthylene (DHN), dibenzofuran (DBF), fluorene (FL), methylfluoranthene (MF), pentachlorophenol (PCP), methyldibenzothiophene (MDT), methyl- phenanthrene (MP), fluoranthene (FA), and pyrene (PY). (Figure 69). Of these compounds, napthalene, dibenzofuran, methydibenzothiophene, pentachlorophenol, and fluorene are not mutagenic in _Salmonella_ (Anderson _et al_., 1971; McCann _et al_., 1975; Pelroy _et al_., 1983), while fluoranthene and methylphenanthrene are strongly mutagenic (Kaden _et al_., 1979; LaVoie _et al_., 1983). Only three compounds remained in detectable levels in the runoff sample collected on day 360. These included pentachlorophenol, fluoranthene, and pyrene (Figure 70). In the runoff water, the impact of reducing the concentration of organic compounds on the mutagenic potential of the mixture is difficult to predict because mixtures of polycyclic aromatic hydrocarbons have been shown to cause synergistic, additive, and

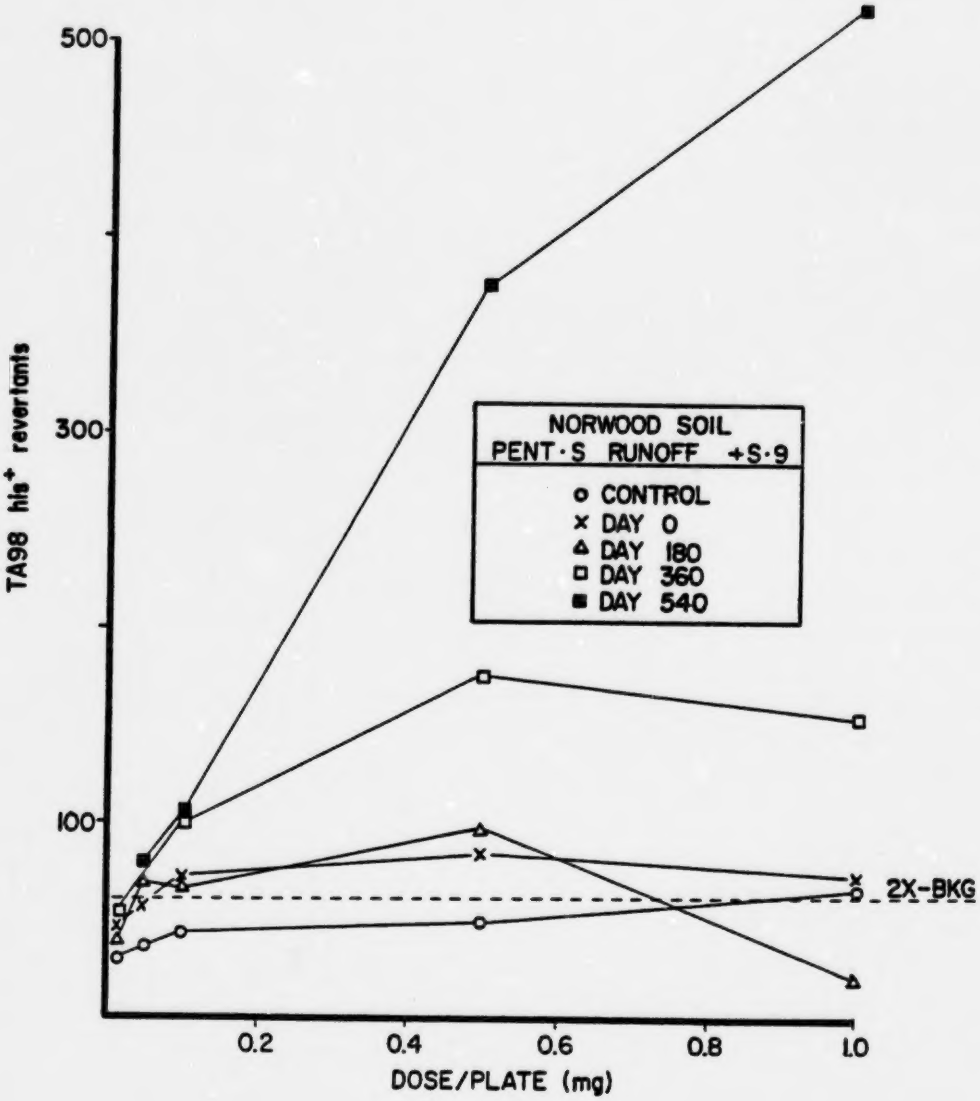


Figure 65. Mutagenic activity with metabolic activation of runoff water from PENT S amended Norwood soil.

TABLE 51. MUTAGENIC ACTIVITY, AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 OF RUNOFF WATER FROM PENT S AMENDED NORWOOD SOIL

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
BKG	-	20 ± 1.9	NT*	17 ± 1.7	21 ± 2.6	11 ± 11.8	NT
	+	29 ± 8.0	NT	33 ± 3.9	24 ± 8.1	31 ± 5.2	NT
0	-	21 ± 8.3	Toxic	Toxic	18 ± 1.3	17 ± 2.1	16 ± 3.6
	+	30 ± 4.3	65 ± 17.1	87 ± 10.1	76 ± 10.6	58 ± 7.6	45 ± 17.0
180	-	26 ± 4.6	Toxic	22 ± 20.0	31 ± 2.0	32 ± 3.5	26 ± 5.3
	+	36 ± 7.9	20 ± 35.2	98 ± 12.1	60 ± 15.0	65 ± 16.7	46 ± 13.9
360	-	25 ± 3.3	47 ± 11.5	68 ± 13.3	34 ± 6.1	36 ± 7.6	27 ± 1.5
	+	42 ± 5.4	153 ± 47.3	175 ± 65.5	99 ± 38.4	77 ± 22.6	50 ± 2.9
540	-	31 ± 4.3	166 ± 92.6	193 ± 116.1	82 ± 49.0	55 ± 22.8	27 ± 6.4
	+	39 ± 3.7	514 ± 404.2	373 ± 232.0	108 ± 35.6	72 ± 15.3	50 ± 2.3

* NT = not tested.

TABLE 52. MUTAGENIC ACTIVITY, AS MEASURED WITH S. TYPHIMURIUM STRAIN TA100 OF RUNOFF WATER FROM PENT S AMENDED NORWOOD SOIL

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
BKG	-	104 ± 16.7	NT*	51 ± 60.0	56 ± 65.3	52 ± 58.8	NT
	+	81 ± 35.4	NT	82 ± 24.7	76 ± 17.4	73 ± 25.7	NT
0	-	87 ± 34.4	Toxic	Toxic	82 ± 8.7	81 ± 10.0	79 ± 7.2
	+	101 ± 10.2	127 ± 27.5	261 ± 48.0	288 ± 29.5	208 ± 27.5	97 ± 28.1
180	-	119 ± 8.8	27 ± 47.9	63 ± 54.9	106 ± 18.3	108 ± 16.6	115 ± 10.4
	+	111 ± 16.4	55 ± 95.8	310 ± 47.7	198 ± 60.7	239 ± 49.7	143 ± 21.1
360	-	167 ± 17.0	25 ± 42.7	126 ± 35.2	158 ± 6.0	155 ± 10.8	149 ± 14.1
	+	147 ± 7.6	283 ± 106.5	501 ± 202.6	378 ±238.2	313 ± 177.1	179 ± 20.6
540	-	121 ± 9.5	92 ± 25.5	124 ± 49.2	112 ± 2.1	106 ± 13.7	100 ± 9.5
	+	120 ± 7.0	306 ± 82.1	486 ± 115.0	273 ± 79.2	198 ± 28.6	130 ± 5.7

* NT = not tested.

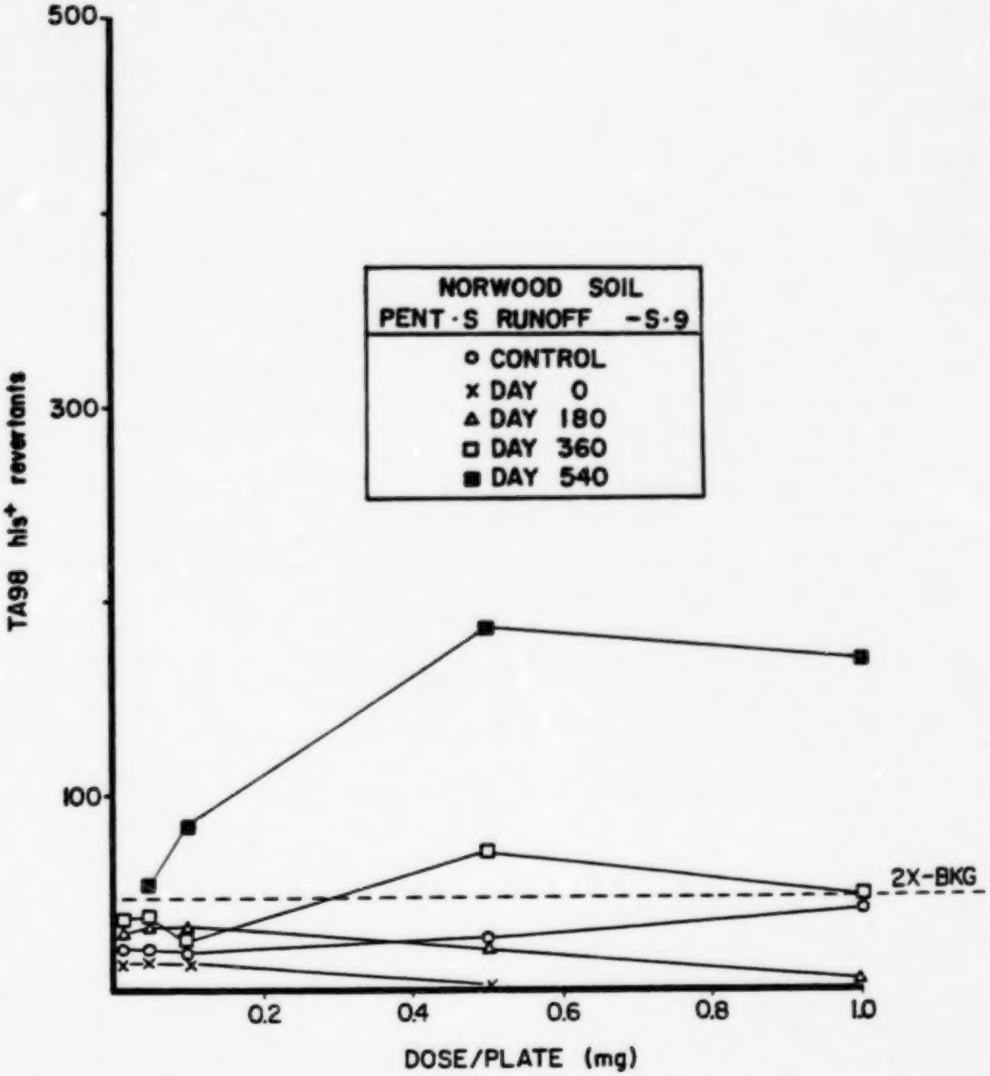


Figure 66. Mutagenic activity without metabolic activation of runoff water from PENT S amended Norwood soil.

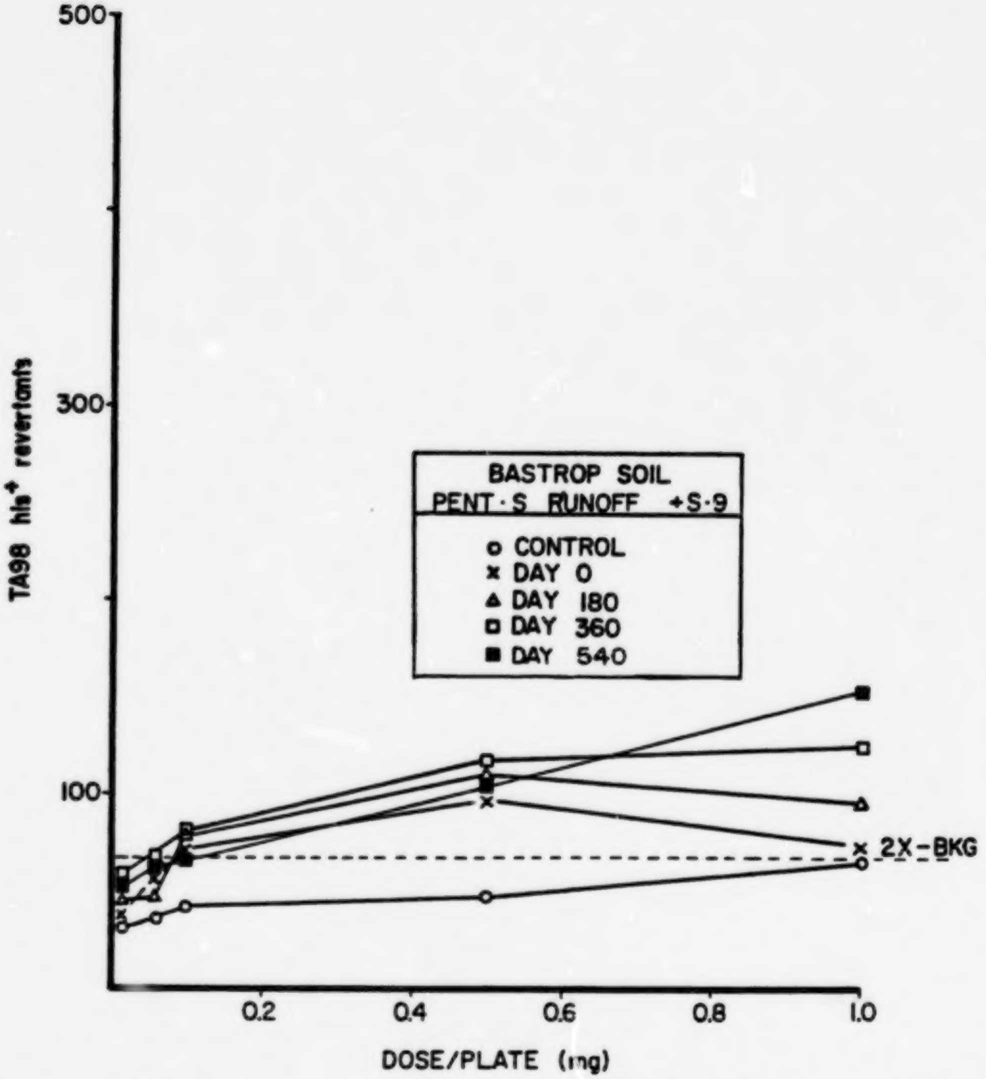


Figure 67. Mutagenic activity with metabolic activation of runoff water from PENT S amended Bastrop soil.

TABLE 53. MUTAGENIC ACTIVITY, AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 OF RUNOFF WATER FROM PENT S AMENDED BASTROP SOIL

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
BKG	-	20 ± 1.9	24 ± 2.6	23 ± 4.7	20 ± 4.8	22 ± 5.0	NT*
	+	29 ± 8.0	31 ± 9.3	27 ± 28.7	27 ± 3.5	30 ± 8.2	NT
0	-	23 ± 2.3	Toxic	15 ± 2.2	18 ± 5.4	20 ± 1.0	22 ± 6.2
	+	28 ± 4.7	68 ± 27.1	92 ± 8.4	69 ± 19.4	57 ± 15.6	34 ± 5.8
180	-	24 ± 4.1	30 ± 16.7	46 ± 30.3	27 ± 8.0	22 ± 5.9	22 ± 2.6
	+	32 ± 6.1	103 ± 21.7	112 ± 4.2	72 ± 2.1	49 ± 8.3	42 ± 3.5
360	-	34 ± 7.8	50 ± 7.1	42 ± 7.5	35 ± 3.0	31 ± 3.2	31 ± 3.5
	+	47 ± 6.2	122 ± 5.9	114 ± 4.9	70 ± 6.1	62 ± 5.0	55 ± 3.5
540	-	27 ± 4.1	71 ± 33.9	52 ± 18.4	31 ± 5.7	26 ± 6.4	26 ± 1.4
	+	39 ± 3.7	151 ± 18.4	102 ± 2.8	68 ± 7.8	59 ± 5.7	53 ± 3.5

* NT = not tested.

TABLE 54. MUTAGENIC ACTIVITY, AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA100 OF RUNOFF WATER FROM PENT S AMENDED BASTROP SOIL

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
BKG	-	104 ± 17.1	NT*	56 ± 63.0	50 ± 56.9	51 ± 57.9	NT
	+	88 ± 19.5	83 ± 17.3	79 ± 41.5	86 ± 34.9	86 ± 19.9	NT
0	-	88 ± 10.6	Toxic	Toxic	84 ± 9.5	73 ± 12.6	77 ± 3.9
	+	104 ± 9.0	253 ± 20.6	206 ± 27.1	239 ± 32.5	202 ± 27.0	112 ± 20.7
180	-	107 ± 18.5	Toxic	120 ± 16.5	96 ± 13.6	105 ± 21.6	101 ± 11.3
	+	99 ± 16.7	86 ± 81.0	304 ± 60.12	268 ± 11.8	183 ± 25.5	103 ± 8.6
360	-	126 ± 10.7	75 ± 6.8	121 ± 8.3	122 ± 8.4	112 ± 9.5	113 ± 9.2
	+	130 ± 13.1	323 ± 13.2	394 ± 51.0	209 ± 6.1	166 ± 14.6	139 ± 7.5
540	-	136 ± 12.6	99 ± 31.8	141 ± 24.0	141 ± 11.3	132 ± 16.3	127 ± 4.9
	+	142 ± 15.4	384 ± 74.2	318 ± 62.9	209 ± 22.6	163 ± 2.8	144 ± 30.4

* NT = not tested.

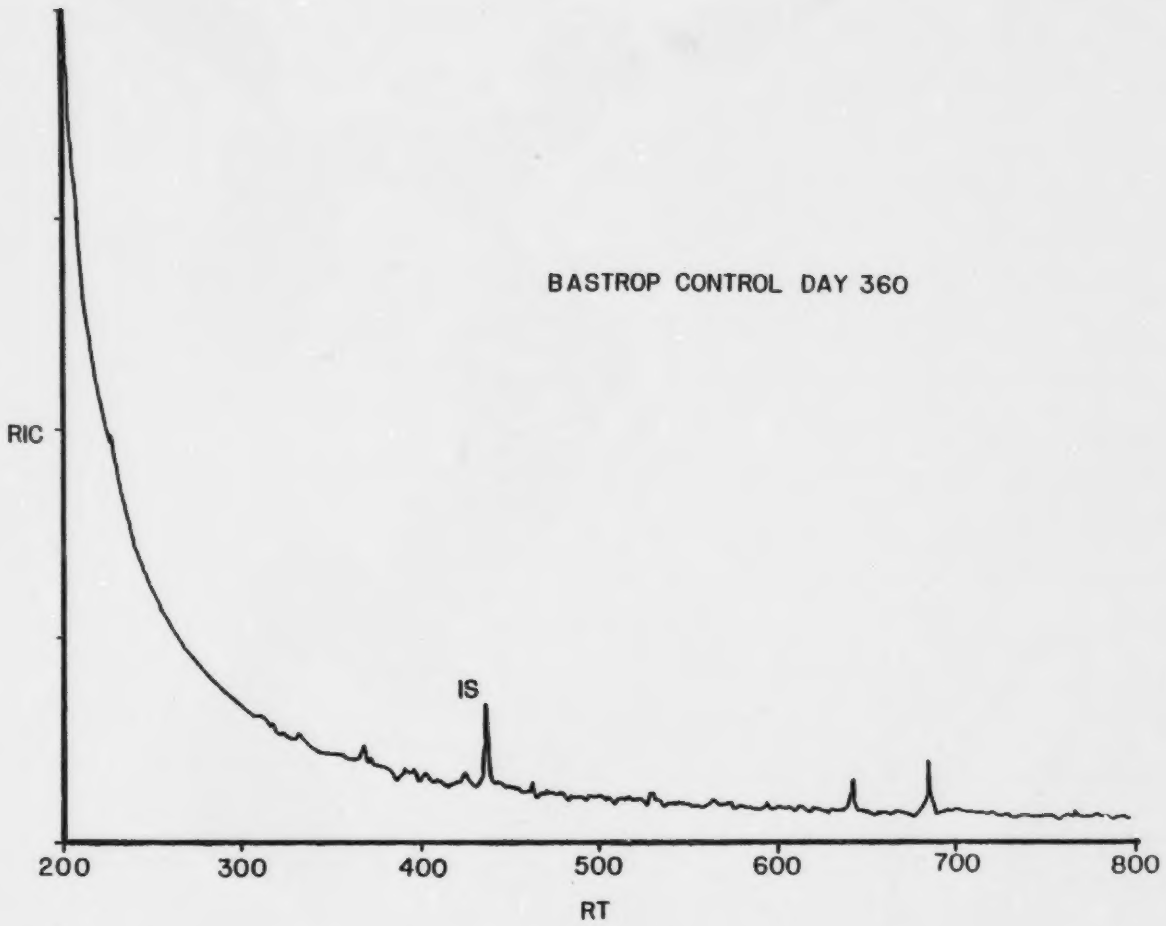


Figure 68. GC/MS chromatograph of organic extract of runoff water from unamended Bastrop soil collected on day 360.

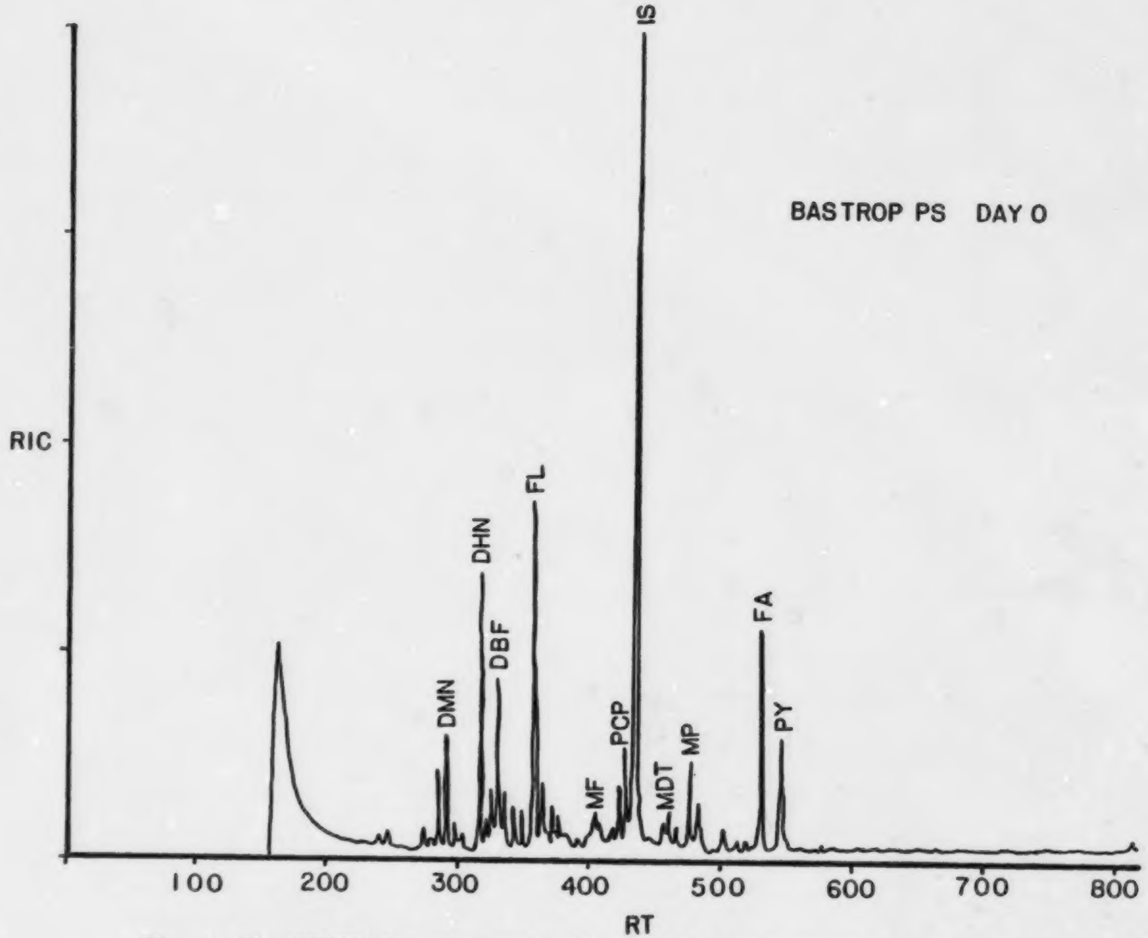


Figure 69. GC/MS chromatograph of organic extract of runoff water from PENT S amended Bastrop soil collected on day 0.

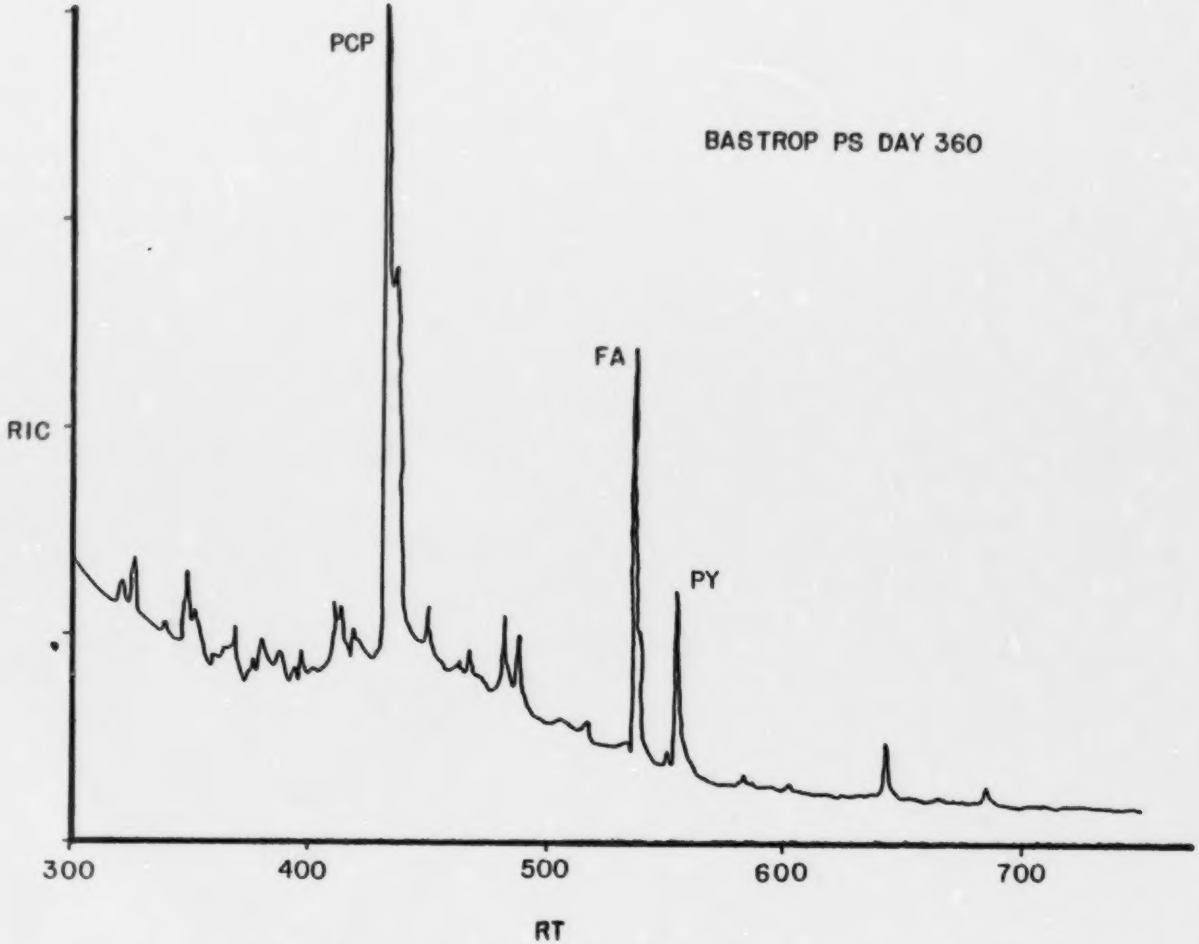


Figure 70. GC/MS chromatograph of organic extract of runoff water from PENT S amended Bastrop soil collected on day 360.

antagonistic responses (Hass et al., 1981; Haugen and Peak, 1983). A comparison of the runoff samples collected on day 540 indicates that greater amounts of mutagenic activity were detected in the runoff water from the Norwood soil than in the runoff water from the Bastrop soil (Tables 51 and 53). The results from chemical and biological analysis of runoff water from the PENT S amended soils indicate that the mutagenic activity of the compounds residual on day 540 was greater than the level of mutagenicity present in the more complex samples collected prior to day 540.

The results from the biological analysis of the runoff water from SWRI waste amended soils are provided in Figures 71 and 72, and Tables 55 through 58. For strain TA98, the mutagenic activity of the residual hydrocarbons in the runoff water from the Norwood soil increased from day 0 through day 360 (Table 55); whereas, for strain TA100 the mutagenicity increased from day 0 to 180 and decreased slightly on day 360 (Table 56).

The mutagenic potential of the runoff water from the Bastrop soils amended with the SWRI waste showed a similar trend. The net TA98 his$^+$ revertants with metabolic activation at a dose level of 500 μg/plate on day 0, 180 and 360 was 86, 88, and 206, respectively. Without activation, the net TA98 his$^+$ revertants at the same dose level were 21, 25, and 196 on days 0, 180, and 360, respectively (Table 57). In strain TA100, the net his$^+$ revertants at 500 μg/plate with metabolic activation increased from day 0 to 180 and decreased from day 180 to 360 (Table 58). The runoff water collected on day 0, 180, and 360 from both soils yielded consistently similar dose-response curves when the total mutation frequencies in strain TA98 with metabolic activation are compared (Figures 71 and 72). In both soils, the mutagenic potential of the residual hydrocarbons in the runoff water increased slightly from day 0 to 180 and almost doubled from day 180 to 360. In addition, direct-acting mutagens were detected in the samples collected on day 360, whereas only promutagens were detected on days 0 and 180.

The mutagenic potential of the runoff water from the combined API separator/slop-oil emulsion solids (COMBO) waste amended soil did not follow a consistent trend in either soil. In the Norwood soil, the mutagenic activity of the residual hydrocarbons decreased from day 0 to 180, and then increased from day 180 to 360 (Figure 73). The values for the net TA98 his$^+$ revertants with metabolic activation at a dose level of 500 μg/plate were 95, 40, and 144 on day 0, 180, and 360, repectively (Table 59). In strain TA100, none of the runoff samples from the COMBO waste amended Norwood soil induced a positive response (Table 60). The runoff water collected from the COMBO waste amended Bastrop soil on day 0 induced a positive response only at the highest dose level in strain TA98 with metabolic activation (Table 61 and Figure 74). Using the modified two-fold rule (Chu et al., 1981), this sample would be considered non-mutagenic. However, the mutagenic activity induced by the residual hydrocarbons in the runoff water from the COMBO waste amended

TABLE 55. MUTAGENIC ACTIVITY, AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 OF RUNOFF WATER FROM NORWOOD SOILS AMENDED WITH SWRI WASTE

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
0	-	23 ± 2.8	37 ± 8.5	35 ± 4.0	30 ± 8.3	28 ± 5.3	29 ± 2.2
	+	28 ± 7.4	87 ± 9.2	102 ± 39.4	82 ± 10.2	67 ± 6.8	38 ± 15.0
180	-	30 ± 4.1	65 ± 1.0	52 ± 1.0	38 ± 3.2	32 ± 0.6	30 ± 1.7
	+	50 ± 4.6	64 ± 12.3	148 ± 15.0	71 ± 14.5	57 ± 4.0	53 ± 8.0
360	-	24 ± 1.6	197 ± 106.0	123 ± 48.0	53 ± 12.0	37 ± 7.0	26 ± 2.1
	+	39 ± 4.2	507 ± 235.1	271 ± 152.8	90 ± 48.5	57 ± 14.3	43 ± 3.0

TABLE 56. MUTAGENIC ACTIVITY, AS MEASURED WITH S. TYPHIMURIUM STRAIN TA100 OF RUNOFF WATER FROM NORWOOD SOILS AMENDED WITH SWRI WASTE

Day	S9	Dose/Plate (mg) 0	1	.5	.1	.05	.01
0	-	114 ± 9.2	Toxic	122 ± 32.8	101 ± 8.5	91 ± 17.4	112 ± 26.0
	+	97 ± 11.9	Toxic	203 ± 31.4	189 ± 26.8	156 ± 10.0	109 ± 4.2
180	-	128.± 15.0	167 ± 12.2	159 ± 17.6	145 ± 19.1	157 ± 3.5	149 ± 2.5
	+	158 ± 11.0	342 ± 21.8	301 ± 10.5	207 ± 36.7	172 ± 9.2	162 ± 6.0
360	-	126 ± 14.3	256 ± 120.0	201 ± 62.9	158 ± 17.9	145 ± 12.7	134 ± 5.1
	+	123 ± 8.1	328 ± 119.4	220 ± 66.4	144 ± 10.6	128 ± 16.4	125 ± 17.2

TABLE 57. MUTAGENIC ACTIVITY, AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 OF RUNOFF WATER FROM BASTROP SOILS AMENDED WITH SWRI WASTE

Day	S9	Dose/Plate (mg) 0	1	.5	.1	.05	.01
0	-	23 ± 5.2	NT*	44 ± 4.4	22 ± 6.3	22 ± 4.6	24 ± 6.2
	+	25 ± 10.2	NT	111 ± 28.7	60 ± 12.9	46 ± 3.5	37 ± 14.2
180	-	31 ± 5.6	65 ± 3.7	56 ± 9.8	40 ± 1.2	37 ± 5.6	30 ± 2.6
	+	45 ± 3.5	64 ± 12.3	133 ± 25.1	62 ± 1.2	57 ± 1.5	47 ± 8.5
360	-	32 ± 4.6	306 ± 184.5	228 ± 118.1	75 ± 37.2	56 ± 23.2	33 ± 8.9
	+	43 ± 3.5	475 ± 218.6	249 ± 131.1	76 ± 15.0	51 ± 5.7	46 ± 0.6

* NT = not tested.

TABLE 58. MUTAGENIC ACTIVITY, AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA100 OF RUNOFF WATER FROM BASTROP SOILS AMENDED WITH SWRI WASTE

Day	S9	Dose/Plate (mg) 0	1	.5	.1	.05	.01
0	-	114 ± 9.3	Toxic	104 ± 10.7	102 ± 10.5	118 ± 15.5	116 ± 12.4
	+	97 ± 12.0	Toxic	90 ± 103.3	55 ± 62.0	41 ± 46.1	46 ± 52.1
180	-	128 ± 15.0	169 ± 12.3	163 ± 4.6	141 ± 11.4	147 ± 9.0	147 ± 3.2
	+	158 ± 11.0	304 ± 7.6	276 ± 17.6	196 ± 41.6	163 ± 14.2	168 ± 6.1
360	-	126 ± 14.0	180*	172	143	137	126
	+	140 ± 16.0	254	216	166	174	157

* Mean represents average of only two values; standard deviation not provided.

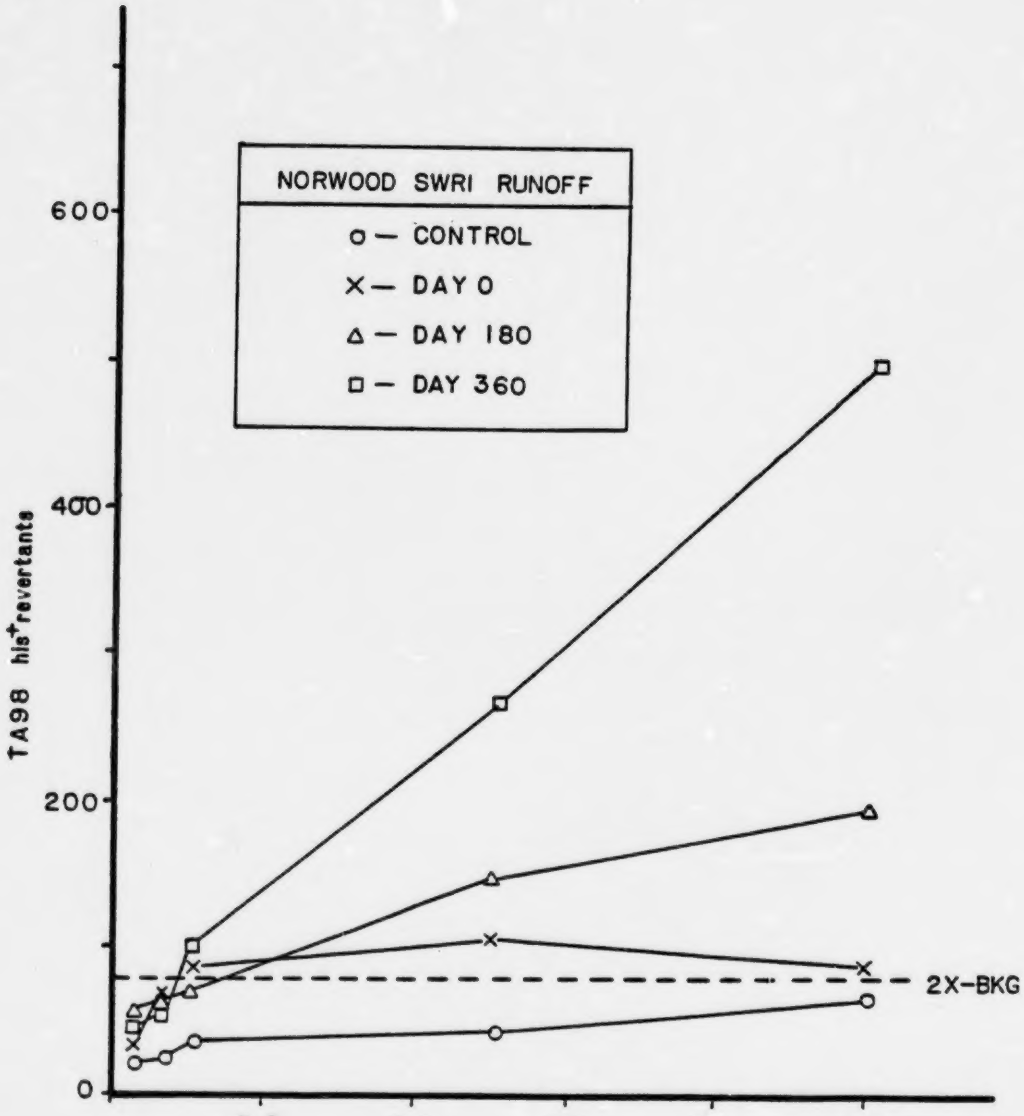


Figure 71. Mutagenic activity with metabolic activation of runoff water from SWRI amended soil.

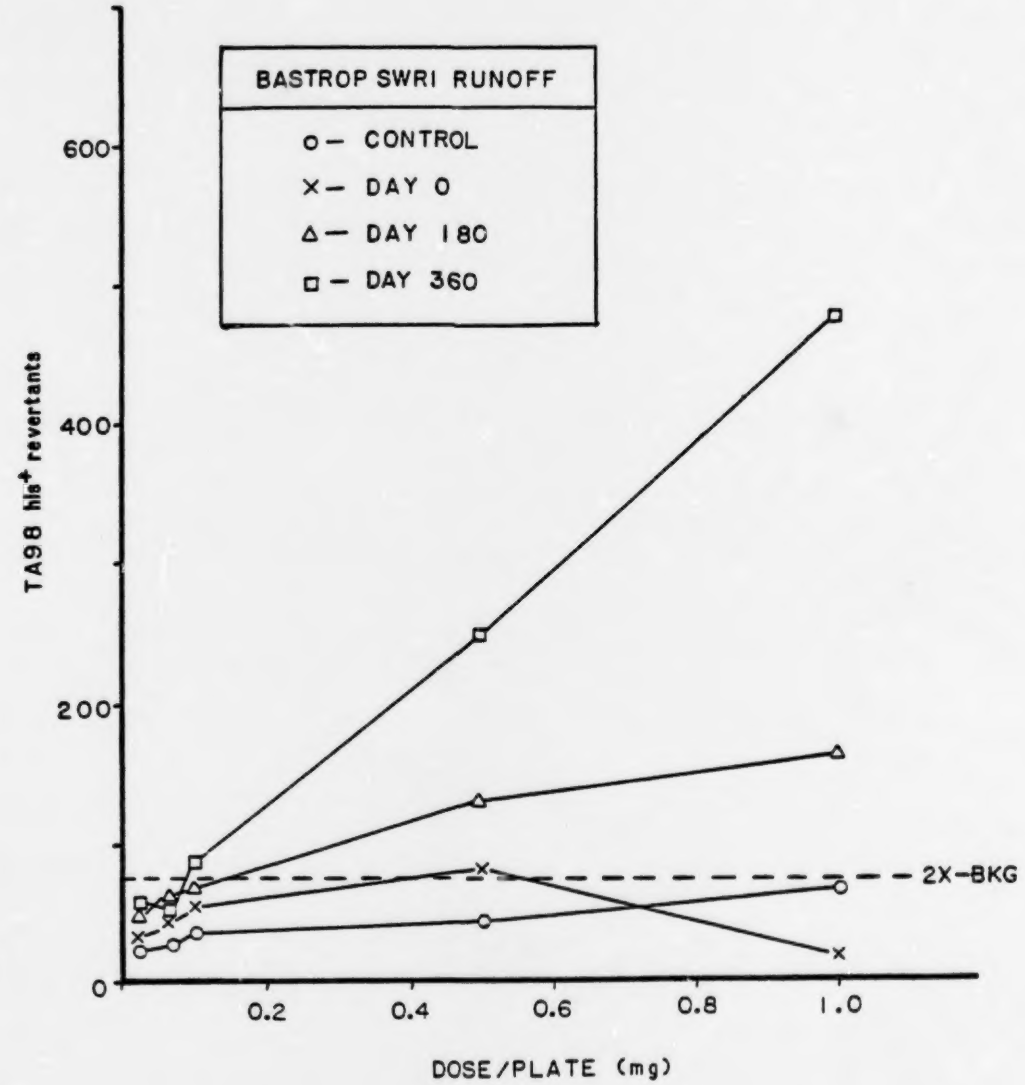


Figure 72. Mutagenic activity with metabolic activation of runoff water from SWRI amended Bastrop soil.

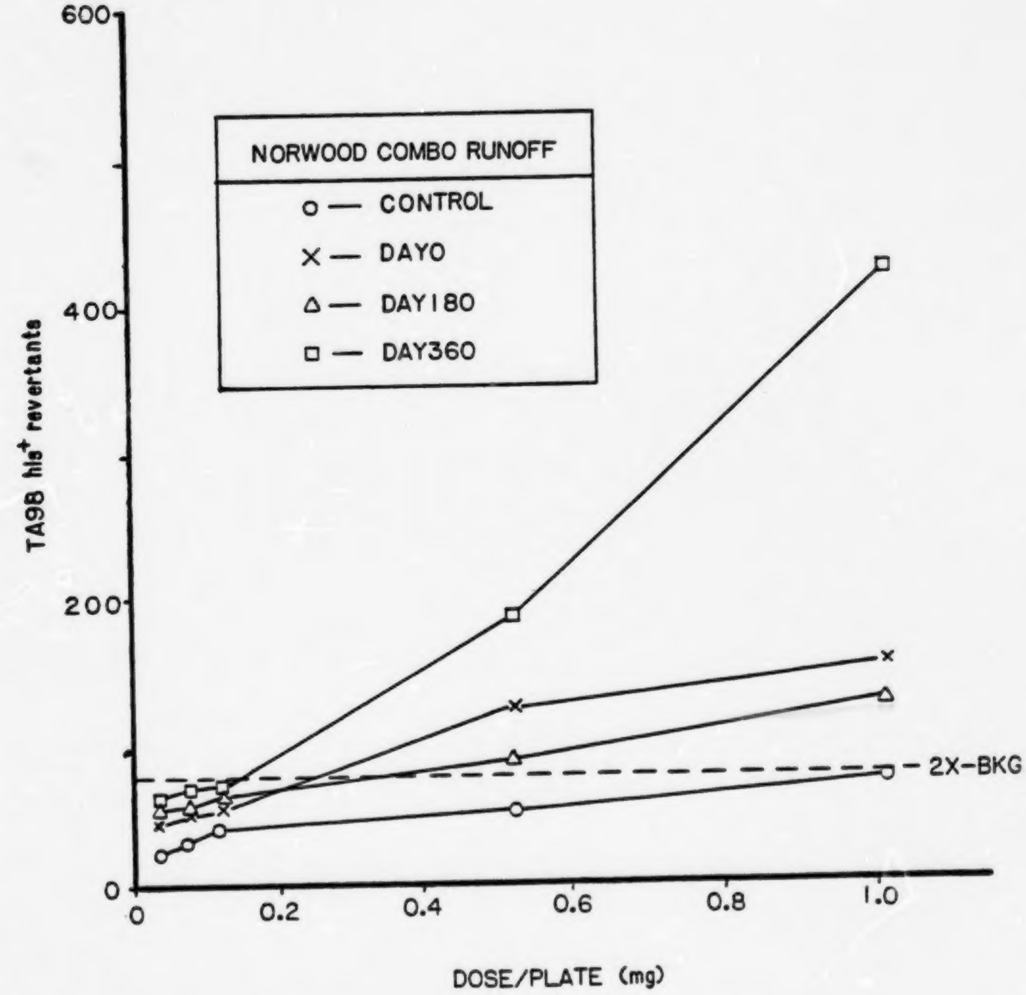


Figure 73. Mutagenic activity with metabolic activation of runoff water from COMBO amended Norwood soil.

TABLE 59. MUTAGENIC ACTIVITY, AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA98 OF RUNOFF WATER FROM NORWOOD SOILS AMENDED WITH COMBO WASTE

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
0	-	22 ± 5.4	NT*	61 ± 22.9	30 ± 11.1	33 ± 16.9	34 ± 13.2
	+	41 ± 5.8	79 ± 95.7	136 ± 16.6	65 ± 45.5	61 ± 11.9	43 ± 2.9
180	-	35 ± 2.8	54 ± 11.1	43 ± 7.5	33 ± 4.6	30 ± 2.5	30 ± 1.5
	+	44 ± 2.3	124 ± 30.3	84 ± 22.2	62 ± 11.4	50 ± 1.7	47 ± 2.9
360	-	25 ± 5.4	440 ± 50.9	272 ± 43.1	80 ± 16.3	64 ± 11.3	33 ± 4.9
	+	42 ± 1.9	430 ± 4.9	186 ± 44.5	63 ± 1.4	62 ± 9.2	61 ± 8.5

* NT = not tested.

TABLE 60. MUTAGENIC ACTIVITY, AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA 100 OF RUNOFF WATER FROM NORWOOD SOILS AMENDED WITH COMBO WASTE

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
0	-	96 ± 6.0	NT*	119 ± 19.1	114 ± 17.5	102 ± 16.6	109 ± 9.3
	+	114 ± 12.7	144 ± 35.4	122 ± 24.7	136 ± 25.0	111 ± 22.2	83 ± 21.5
180	-	114 ± 18.0	96 ± 35.6	92 ± 30.6	94 ± 35.9	87 ± 31.8	88 ± 34.9
	+	112 ± 11.6	212 ± 15.4	155 ± 36.8	123 ± 12.6	117 ± 4.9	108 ± 6.1
360	-	145 ± 12.8	151 ± 62.9	112 ± 53.7	108 ± 50.2	100 ± 38.2	100 ± 38.2
	+	136 ± 11.1	176 ± 68.6	148 ± 56.6	109 ± 63.6	108 ± 50.2	96 ± 48.1

*NT = not tested.

TABLE 61. MUTAGENIC ACTIVITY, AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA98 OF RUNOFF WATER FROM BASTROP SOILS AMENDED WITH COMBO WASTE

Day	S9	Dose/Plate (mg) 0	1	.5	.1	.05	.01
0	-	27 ± 7.2	39 ± 8.2	39 ± 1.7	23 ± 9.0	35 ± 16.9	30 ± 10.8
	+	45 ± 9.0	86 ± 19.3	60 ± 10.9	50 ± 1.9	37 ± 6.7	38 ± 5.9
180	-	28 ± 4.9	112 ± 52.0	81 ± 28.7	43 ± 8.7	38 ± 2.3	31 ± 2.0
	+	46 ± 4.2	175 ± 9.7	123 ± 14.6	58 ± 4.6	54 ± 3.6	40 ± 2.5
360	-	25 ± 4.9	85 ± 14.6	56 ± 8.5	39 ± 2.1	29 ± 1.2	22 ± 2.5
	+	42 ± 1.7	160 ± 37.2	101 ± 16.1	60 ± 5.0	58 ± 4.6	51 ± 1.7

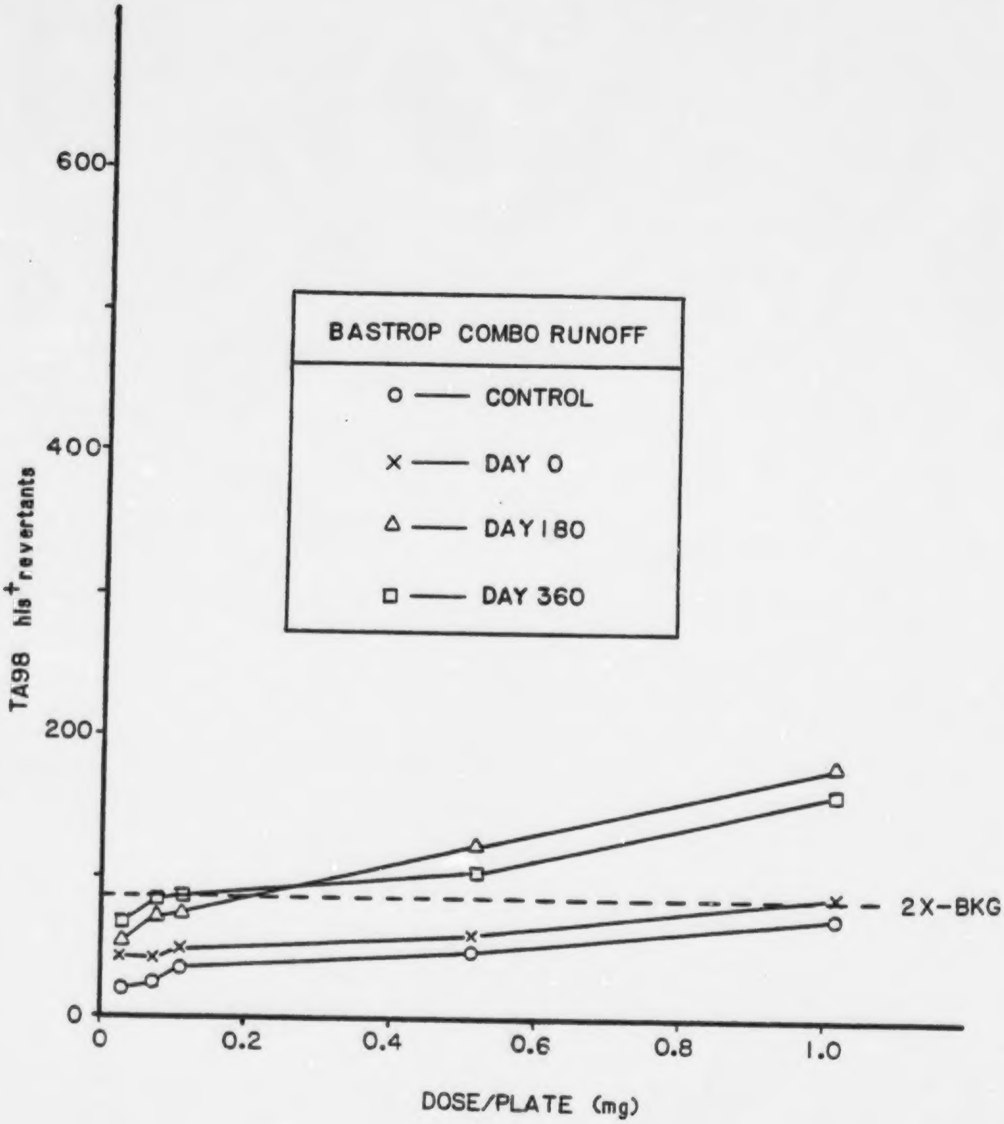


Figure 74. Mutagenic activity with metabolic activation of runoff water from COMBO amended Bastrop soil.

Bastrop soil in strain TA98 with metabolic activation increased from 15 to 77 to 59 net revertants on day 0, 180, and 360, respectively (Table 61). As with the Norwood soil, in strain TA100, none of the runoff samples from the COMBO waste amended Bastrop soil induced a positive response (Table 62). Thus, the mutagenic potential of the residual hydrocarbons in the runoff water from both soils was greater on day 360 than on day 0. In addition, direct-acting mutagens were detected on day 360 but not on day 0.

Degradation, infiltration, and removal will influence the quality and quantity of hazardous organic compounds in runoff water from a land treatment facility. In order to evaluate the influence of these factors on the mutagenic potential of runoff water, the results from the waste amended and control soils were compared on the basis of equivalent volumes. Because of the limited amount of sample available, the extracts of runoff water samples were tested in the Aspergillus assay at volumes approximately one-fourth that tested in the Salmonella assay. While it is unfortunate that equal volumes were not tested in the two bioassays, some general conclusions can be drawn from the results in the different bioassays. A comparison of the results from testing the equivalent of 10 ml of runoff water from the PENT S amended Norwood soil in the Salmonella assay indicates that the mutagenic potential increases consistently from day 0 to 180 to 360 and from day 360 to 540 (Figure 75). Contrasting results were observed in the Aspergillus assay (Figure 76). In the PENT S amended Norwood soil, the mutagenic activity of the runoff water collected on day 360 decreased to a level approximately 16% of that detected on day 0 (Figure 76). The surviving fraction in Aspergillus increased significantly from day 0 to 360. If this effect also occurred in the Salmonella assay, the induced mutants per survivor in Salmonella would be greater on day 0 and approximately the same on day 360; thus, the results from the two bioassays would then be comparable. The observed difference in the results from the two bioassays is probably a reflection of the increased metabolic and DNA repair capabilities of the eukaryotic system (Dunkel, 1981).

In the Bastrop soil, the mutagenic response in both bioassays decreased from day 0 to 180 and from day 180 to 360 (Figures 76 and 77). In both bioassays, the response induced by the sample collected on day 360 was at a level that was only slightly greater than twice background. However, on day 540 there was an appreciable increase in both the amount of extractable hydrocarbon and the mutagenic potential of the runoff water from the PENT S amended Bastrop soil in the Salmonella assay. The results obtained from the runoff water from either soil amended with the PENT S waste as measured with the Salmonella and Aspergillus assays indicate that significant levels of mutagenic activity are detectable 360 days after waste application. However, the overall results indicate that the mutagenic potential of the runoff water collected on day 360 is less than on day 0.

The runoff water from soils amended with the SWRI and COMBO waste

TABLE 62. MUTAGENIC ACTIVITY, AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA100 OF RUNOFF WATER FROM BASTROP SOILS AMENDED WITH COMBO WASTE

		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
0	-	96 ± 6.0	100 ± 19.4	102 ± 16.5	95 ± 13.6	105 ± 12.9	95 ± 18.7
	+	114 ± 12.7	173 ± 10.8	172 ± 3.6	140 ± 20.7	131 ± 14.9	99 ± 10.8
180	-	109 ± 13.9	NT*	NT	NT	NT	NT
	+	105 ± 16.5	NT	NT	NT	NT	NT
360	-	150 ± 10.3	150 ± 17.7	143 ± 9.5	145 ± 4.5	138 ± 7.5	135 ± 4.0
	+	139 ± 9.0	209 ± 11.1	172 ± 5.5	145 ± 9.1	148 ± 4.2	130 ± 8.0

*NT = not tested.

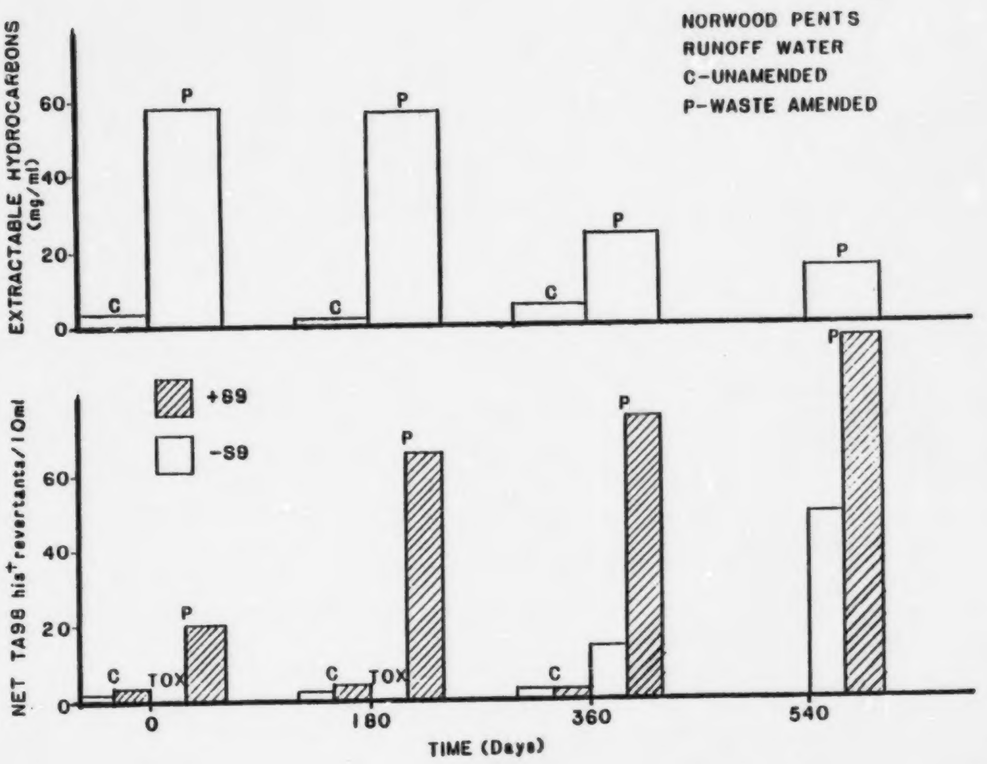


Figure 75. Extractable hydrocarbons (mg/l) and mutagenic activity (revertants/10 ml) in runoff water from PENT S amended Norwood soil.

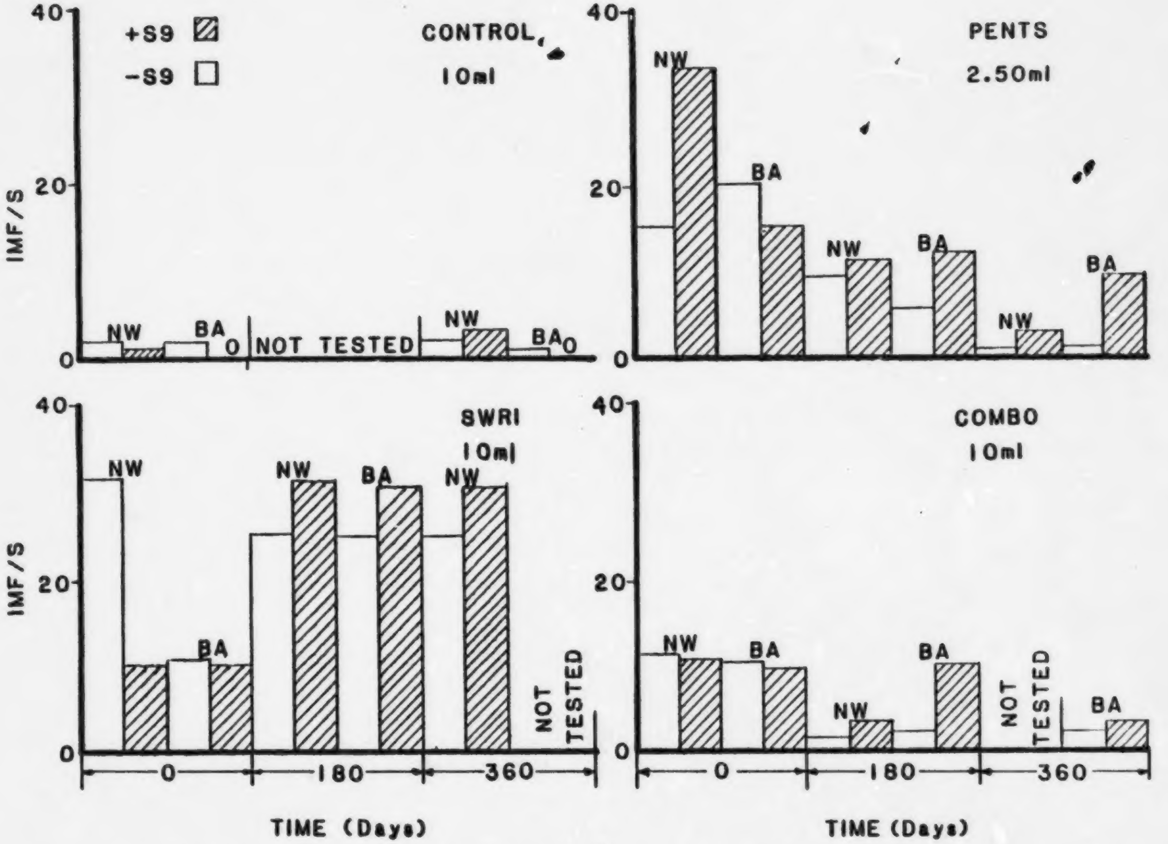


Figure 76. Total mutation frequency per 10^6 survivors in *A. nidulans* induced by the extractable hydrocarbons in runoff water from waste amended soils.

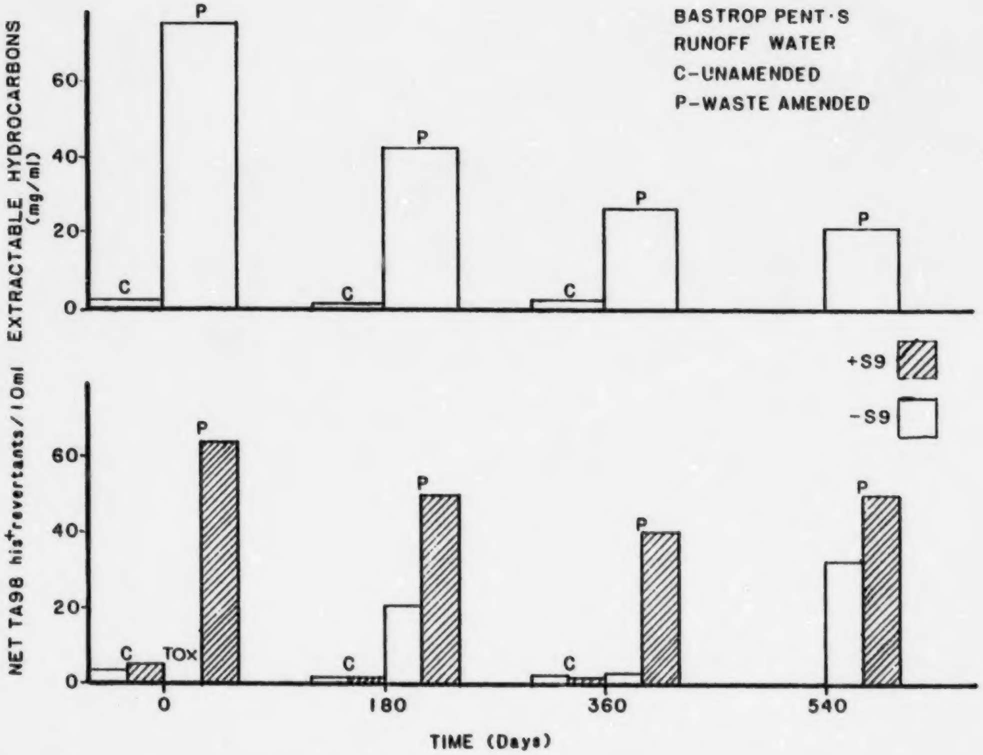


Figure 77. Extractable hydrocarbons (mg/l) and mutagenic activity (revertants/10 ml) in runoff water from control and PENT S amended Bastrop soils.

contained lower levels of hydrocarbons than did the water from the PENT S waste (Table 63). Therefore, the quality of runoff water from soils amended with the SWRI or COMBO waste were compared on the basis of a 50 ml volume. The amount of extractable hydrocarbons in runoff water from SWRI waste amended Norwood and Bastrop soils was less than that in runoff water from unamended soils on day 0. On day 180, the level of extractable hydrocarbons in the runoff water increased approximately ten-fold and then decreased by approximately 50% on day 360. The mutagenic potential of 50 ml of runoff water from SWRI waste amended Norwood and Bastrop soils was non-mutagenic on day 0, increased to 3 to 4 times background on day 180, and increased still further on day 360 (Figures 78 and 79). In addition, while greater amounts of mutagenic activity were detected with metabolic activation, the mutagenic activity in the absence of metabolic activation also increased significantly from day 0 to 180 and from day 180 to 360. The results from the *Aspergillus* assay followed the same general trend. With metabolic activation, the mutagenic potential of the sample collected on day 180 was approximately three times that of the sample from day 0 (Figure 76). In addition, the mutagenic potential of the sample from day 180 was essentially the same as that of the same from day 360. Thus, the mutagenic activity of the runoff water from both the Norwood and Bastrop soils amended with the SWRI waste followed the same general trend increasing from day 0 to 180 and from day 180 to 360. In addition, slightly greater levels of mutagenic activity were detected in the runoff water from the Norwood soil than from the Bastrop soil.

The level of extractable hydrocarbons in the runoff water from the Norwood soil amended with the COMBO waste decreased by approximately 25% from day 0 to 180 and by approximately 33% from day 180 to 360 (Table 63). The level of mutagenic activity in 50 ml of runoff water decreased by approximately 50% from day 0 to 180 but increased appreciably from day 180 to 360 (Figure 80). The runoff sample collected on day 180 from the COMBO waste amended Norwood soil induced 10 net revertants/50 ml without and 50 net revertants/50 ml with metabolic activation; while, the sample collected on day 360 induced 220 net revertant colonies/50 ml without and 130 net revertants/50 ml with metabolic activation. Similarly, the response obtained in the *Aspergillus* assay with activation decreased by approximately 40% from day 0 to 180, while the sample from day 360 was not tested (Figure 76). In the COMBO waste amended Bastrop soil, none of the runoff samples induced a significant increase in revertant colonies in the absence of metabolic activation. In the presence of metabolic activation, the mutagenic activity in *Salmonella* of the sample collected on day 0 was less than the control, while the mutagenic activity of the sample collected on day 180 was slightly greater than two times background; the sample collected on day 360 induced a response that was greater than 2.5 times background (Figure 81). However, in the *Aspergillus* assay with activation, the mutagenic potential of the runoff water from the Bastrop soil was approximately equal on days 0 and 180 and significantly lower on day 360 (Figure 76). Thus, the results from the *Salmonella* assay indicate that

TABLE 63. TOTAL HYDROCARBONS EXTRACTED FROM RUNOFF WATER USING COMBINED XAD2 RESINS

Sample	Extractable Hydrocarbons (mg/l) Days After Appplication 0	180	360	540
Wood-Preserving Waste				
Norwood soil	60	59 ± 38	26 ± 12	17 ± 16
Bastrop soil	78	45 ± 5	28 ± 5	36 ± 12
Storm-Water Runoff Impoundment				
Norwood soil	1	13 ± 2	8 ± 1	NS*
Bastrop soil	1	9 ± 3	5 ± 2	NS
Combined API-Separator/ Slop-Oil Emulsion Solid				
Norwood soil	22	15 ± 10	9 ± 5	NS
Bastrop soil	8	5 ± 2	8 ± 2	NS

*No sample collected.

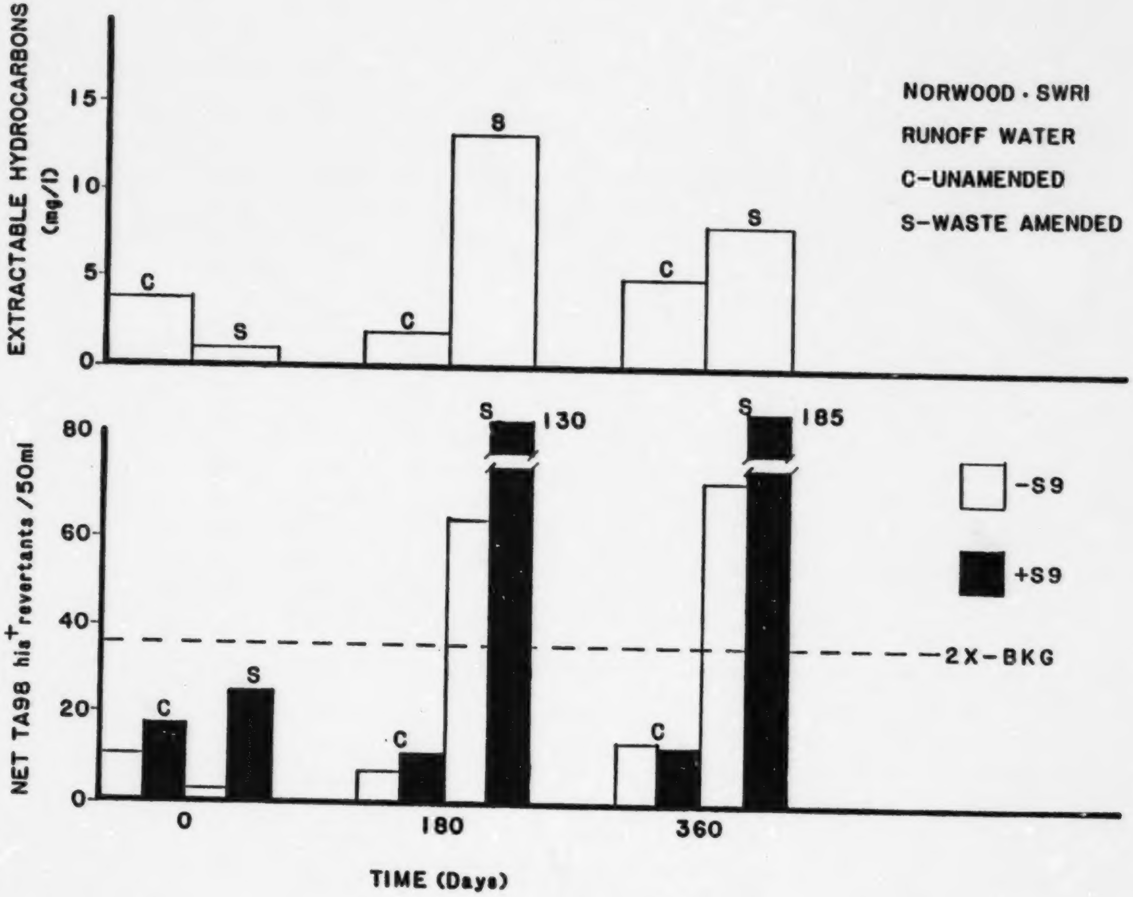


Figure 78. Extractable hydrocarbons (mg/l) and mutagenic activity (revertants /50ml) in runoff water from control and SWRI amended Norwood soils.

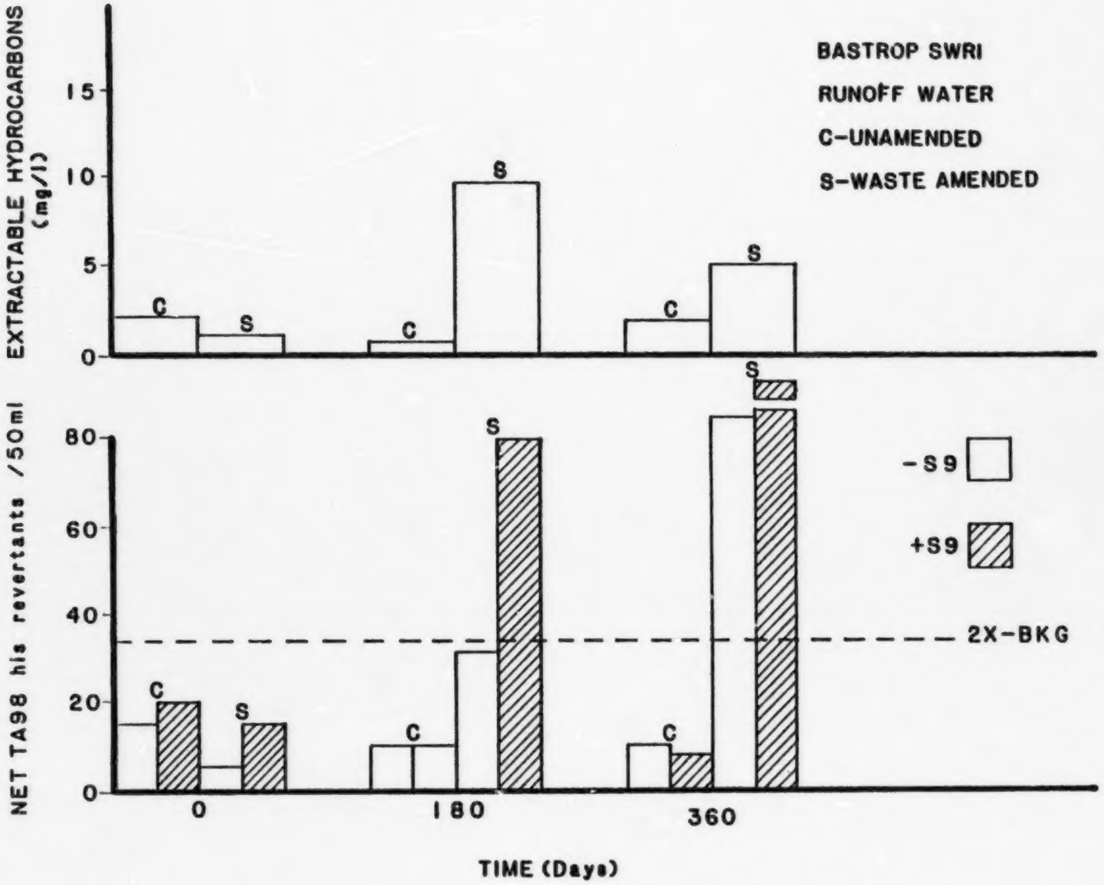


Figure 79. Extractable hydrocarbons (mg/l) and mutagenic activity (revertants/50ml) in runoff water from control and SWRI amended Bastrop soils.

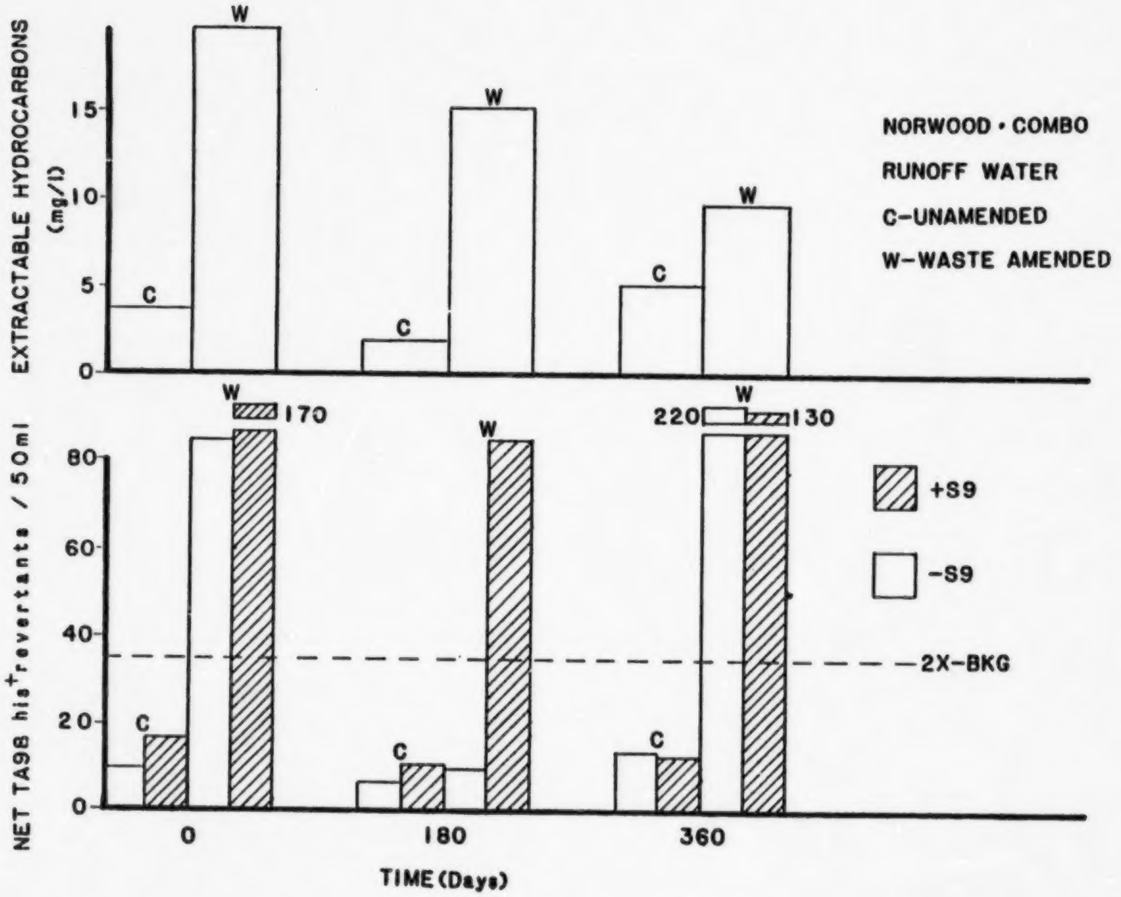


Figure 80. Extractable hydrocarbons (mg/l) and mutagenic activity (revertants/50 ml) in runoff water from control and COMBO amended Norwood soils.

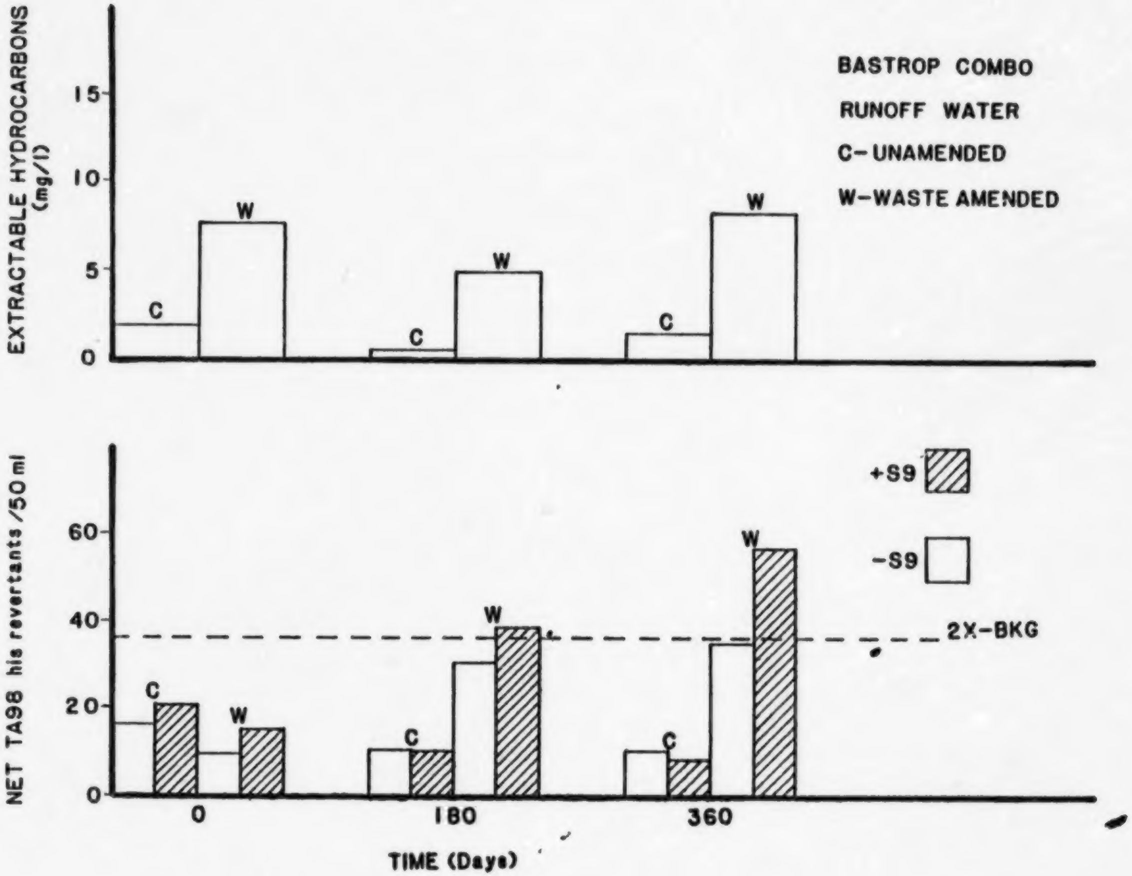


Figure 81. Extractable hydrocarbons (mg/l) and mutagenic activity (revertants/50ml) in runoff water from control and COMBO amended Bastrop soils.

for COMBO waste amended soils, significantly greater quantities of mutagenic activity were detected in the runoff water from the Norwood soil than in the runoff water from the Bastrop soil; and in both soils, the maximum amount of mutagenic activity was observed in the sample collected 360 days after waste application.

The results from this segment of the research indicate that the mutagenic potential of runoff water from hazardous waste amended soils should eventually return to background levels. The amount of time required for the mutagenic potential of runoff water to return to background levels will be regulated by the quantity and degradation rate of mutagenic compounds in the waste as well as the waste loading rate. These results have also demonstrated that there will be dramatic differences in the degradation rates between wastes and between soils. As a general rule, the mutagenic potential of the runoff water from day 0 was greatest from the PENT S amended soils and least from the COMBO waste amended soils; while the mutagenic potential of the sample from the SWRI waste amended soil was greatest on day 180 and 360. In addition, the mutagenic potential of runoff water from waste amended Norwood soil was greater than that of the runoff water from the Bastrop soil. Thus, since different soils will have substantially different capacities to retain and degrade organic compounds, bioassays provide the most effective analytical tool for evaluating when runoff water may be safely released following closure of a hazardous waste land treatment facility.

SECTION 9

AFFECT OF DEGRADATION ON THE MUTAGENIC ACTIVITY OF HAZARDOUS WASTE AMENDED SOIL

INTRODUCTION

The application of hazardous waste to soil is restricted by regulations to include only those wastes which will be rendered less or non-hazardous by chemical or biological reactions in the soil (EPA, 1982). These regulations assume that degradation, immobilization, and transformation will serve to reduce the hazardous characteristics of soil applied waste constituents. Degradation in soil is generally assumed to be biological, and biological degradation normally accounts for approximately 80% of soil degradation (Hamaker, 1971). However, in a soil contaminated by the toxic and resistant compounds frequently encountered in a hazardous waste, chemical and photochemical degradation will also play an important role. Soil degradation may not result in the complete mineralization of a hazardous waste, but soil degradation can certainly render waste constituents less or non-hazardous by removing substituted groups, by breaking an aromatic ring, or by substitutions that produce a less reactive product. Soil degradation may not, however, always result in detoxification of a hazardous waste. Thus, techniques are needed to evaluate the affect of degradation on the mutagenic activity of hazardous waste amended soil.

The technique which is currently being used to monitor land treatment employs a chemical analysis to define the products of the various reactions that may occur in the soil. Chemical analysis alone may fail to account for the interactions between soil and waste components and the affect of degradation on these interactions. In addition, the results from a chemical analysis must be extrapolated to estimate the toxicological endpoint in a biological system. The use of bioassay directed chemical analysis as an alternative monitoring technique provides more accurate information from which to obtain a risk assessment. This technique employs a battery of short-term bioassays to define the acute and chronic toxic potential of a sample with a chemical analysis to enhance the result from the biological systems.

MATERIALS AND METHODS

Waste

Three petroleum based sludges were used in the greenhouse study. A general description of sludge characteristics is provided in Table 50, while a more detailed description is included in Section 5. The wood-preserving bottom sediment (PENT S), storm-water runoff impoundment (SWRI), and combined API-separator/slop-oil emulsion solids (COMBO) wastes were applied to the soil at hydrocarbon loading rates of 3.1, 4.5, and 4.5%, respectively. Application rates were calculated using the formula discussed in Section 8.

Soil

The physical and chemical properties of the soils used in the greenhouse study are provided in Table 42, while a more detailed discussion of the Bastrop and Norwood soils is included in Section 6.

Extraction Procedures

Soil and waste samples in the greenhouse study were extracted with dichloromethane. Dichloromethane was selected from a group of solvents to extract the organic fractions of the soil as it consistently provided the greatest extraction efficiency for the types of compounds present in the waste (McGill and Rowell, 1980; Donnelly and Brown, 1981). Twenty-five grams of the waste or waste-soil mixture were blended with six volumes of dichloromethane in a Waring Laboratory blender for 30 sec. This extraction was repeated twice or until the extracting solvent remained colorless. Solvent extractions were then combined and taken to dryness on a Brinkman-Bucci rotary evaporator. The residue from this extraction was partitioned into acid, base, and neutral fractions following the scheme outlined in Figure 12.

Chemical Analysis

A chemical analysis was conducted on selected samples by the USEPA's RS Kerr Environmental Research Laboratory. The procedures used were the same as those discussed in Section 8.

Soil Sample Collection

A composite sample representing approximately 500 g of soil was collected from each box 24 to 48 hours following rainfall events, i.e., before, immediately after, and 0, 45, 90, 180, 360, and 540 days after waste application. Soil samples were composited from 6 to 10 randomly selected plugs, each representing the surface to 17 cm soil. Each sample was stored at $0^{o}C$ in a labeled ziplock bag prior to extraction. In addition, reserve samples from each box and each collection date will be stored for five years following completion of the project.

Biological Analysis

Two different microbial mutagenicity assays were employed to evaluate the capacity of soil extracts to induce genetic damage. The *Salmonella*/microsome assay of Ames *et al*. (1975) was used to monitor the mutagenic activity of concentrated water samples. The *Salmonella* strains were supplied by Dr. Bruce N. Ames (University of California, Berkeley, CA). The procedural methods were the same as Ames *et al*. (1975), except that overnight cultures were prepared by inoculation into 10 ml of Nutrient Broth #2 (KC Biological, Inc., Lenexa, KS) and incubated with shaking for 16 h at 37°C. Water extracts were tested on duplicate plates in two independent experiments in the standard plate incorporation assay at a minimum of 4 dose levels of the sample with and without enzyme activation (0.3 ml rat liver/ml S9 mix) using strains TA98 and TA100. Aroclor 1254 induced rat liver was obtained from Litton Bionetics (Charleston, SC). Positive controls included 2 μg/plate N-methyl-N'nitro-N-nitrosoguanidine (Sigma) for TA100, 25 μg/plate 2-nitrofluorene (Aldrich) for TA98, and 10 μg/plate 2-aminoanthracene (Sigma) which was used with all strains to verify the functioning of the metabolic activation system. All reagents and extracts were tested for sterility; DMSO was used as a negative control.

Soil samples were also tested in the eukaryotic bioassay using *A. nidulans*. The *Aspergillus* system was used to assess the mutagenic potential of soil extracts by evaluating the induction of mutations at the methionine suppressor loci. Conidia from four to five single colonies of the *methG1 biA1* (requiring methionine and biotin) Glasgow strain of *Aspergillus nidulans* grown for 5 to 6 days on a complete medium at 37°C were used for each experiment. Samples were tested at one dose level, usually the extract equivalent to one gram of soil and one exposure time selected to yield approximately 50% survival on five plates in two independent experiments with and without metabolic activation. The procedures were the same as Scott *et al*. (1978) except that the cells were exposed in a 13 x 100 mm screw-capped culture tube. The exposed cells were plated on a complete medium to determine survival and on a methionine-free medium to determine the induced mutation frequency. Mutant colonies were scored after incubation for five days at 37°C. Colonies were divided by colony morphology into three classes, A, B, C, as well as the total mutation frequency represented by the sum of induced mutants from Class A, B, and C. Each of these three classes is believed to involve two genes. The morphology of Class A colonies is green, Class B brown, and Class C green with a white hyaline edge. For the acid, base and neutral fractions of the soil extract, only the total induced mutation frequency was calculated. The frequency of mutations induced by each sample was determined by subtracting the frequency of spontaneous mutations from the total mutation frequency. A sample was considered positive if the induced-mutation frequency was more than twice the spontaneous mutation frequency. Positive controls included 8-methoxypsoralen, 8-methoxypsoralen plus near UV light without activation, and benzo(a)pyrene with metabolic activation.

RESULTS AND DISCUSSION

The soils collected in the greenhouse study were analyzed using both chemical and biological techniques. Residual hydrocarbon analysis was used as a gross measurement of degradation, while GC/MS analysis was used in an attempt to identify the resistant and/or mutagenic waste constituents. Biological analysis was utilized to define the affect of degradation on the mutagenic activity of residual hydrocarbons in the soil and to determine if the wastes were rendered less or non-hazardous by soil incorporation.

The amount of residual hydrocarbons in the soil at various time intervals following waste application provides a preliminary indication of the progress of waste degradation. The total and extractable hydrocarbons in the acid, base, and neutral fractions were determined for each waste/soil mixture on days 0, 90, 180, and 360, and for the PENT S waste/soil mixtures on day 540. The results in Table 64 indicate that the overall degradation rate of the PENT S waste after 540 days in the Norwood and Bastrop soils were 83 and 66%, respectively. The most rapid rate of degradation occurred in the base fraction. In the Norwood soil, only 5% of the base fraction was residual after 540 days, while 10% of the base fraction was residual in the Bastrop soil. The neutral fraction, which represented approximately 90% of the extractable hydrocarbons in the waste, degraded rapidly from day 0 to 180 but slowed appreciably from day 180 to 540. The degradation rate for the neutral fraction over the first 180 days was 66% in the Norwood soil and 51% in the Bastrop soil; whereas, the rate from 180 to 540 was only 50% in the Norwood soil and 25% in the Bastrop soil. These results indicate that significant quantities of the wood-preserving bottom sediment were degraded in both soils. In addition, while less than 50% of the applied hydrocarbons were residual 180 days after waste application, those compounds residual after day 180 had a much slower rate of degradation than the components of the initial waste/soil mixture.

For soils amended with the storm-water runoff impoundment, the most rapid rate of degradation was observed in the Bastrop clay (Table 65). The overall degradation rate of the SWRI waste was 53% in the Norwood soil and 61% in the Bastrop soil. In the Norwood soil, the most rapid rate of degradation after 360 days was 60% in the acid and base fractions. In the Bastrop soil, the most rapid rate of degradation after 360 days was 62%, which occurred in the neutral fraction. While the degradation rates for the first 180 days were quite low in both soils, less than 50% of the applied hydrocarbon remained in the soil after day 360.

The combined API separator/slop-oil emulsion solids exhibited the most rapid rate of degradation of the three wastes studied. The overall degradation rate in the Norwood soil was 74% after 360 days, and 83% in

TABLE 64. TOTAL HYDROCARBONS EXTRACTED FROM SOIL AMENDED WITH WOOD-PRESERVING BOTTOM SEDIMENT

	Extractable Hydrocarbons (mg/g)					% Degraded	
	Days After Appplication						
Sample	0	90	180	360	540	Day 360	Day 540
Norwood Soil							
Acid	7.0	-	4.8 ± 2.1	1.5 ± 0.5	1.6 ± 0.6	79	77
Base	5.8	-	2.7 ± 1.0	0.8 ± 0.15	0.3 ± 0.2	86	95
Neutral	122.2	-	42.1 ± 3.8	34.3 ± 8.4	20.9 ± 3.0	72	83
Total	135.0	-	49.7 ± 6.4	36.7 ± 8.9	23.4 ± 4.5	73	83
Bastrop Soil							
Acid	11.0	6.6 + 2.6	5.3 ± 2.5	4.7 ± 1.1	3.1 ± 0.8	57	72
Base	4.0	5.7 + 0.2	3.1 ± 2.3	1.1 ± 0.3	0.4 ± 0.1	72	90
Neutral	124.0	82.9 + 43.4	60.6 ± 8.7	55.2 ± 5.7	44.3 ± 9.0	55	64
Total	139.0	95.3 + 45.8	69.0 ± 4.4	61.0 ± 6.6	47.8 ± 9.0	56	66

TABLE 65. TOTAL HYDROCARBONS EXTRACTED FROM SOIL AMENDED WITH STORM-WATER RUNOFF IMPOUNDMENT

	Extractable Hydrocarbons (mg/g)				
	Days After Appplication				
Sample	0	90	180	360	% Degraded Day 360
Norwood Soil					
Acid	1	1.5 ± 0.9	0.5 ± 0.3	0.4 ± 0.2	60
Base	0.5	1.0 ± 1.0	0.4 ± 0.15	0.2 ± 0.1	60
Neutral	39.5	32.1 ± 15.5	31.4± 6.9	18.6 ± 3.5	53
Total	41.0	34.7 ± 13.7	32.3± 6.8	19.2 ± 3.7	53
Bastrop Soil					
Acid	0.5	0.7 ± 0.3	0.7 ± 0.1	0.4 ± 0.2	20
Base	0.4	0.3 ± 0.05	0.4 ± 0.2	0.2 ± 0.03	50
Neutral	52.1	33.3 ± 7.9	37.6 ± 11.0	19.9 ± 7.3	62
Total	53.0	34.3 ± 8.1	38.7 ± 10.7	20.5 ± 7.0	61

the Bastrop soil (Table 66). The degradation rates of the fractions of the COMBO waste were 78, 75, and 74% for the acid, base, and neutral fractions in the Norwood soil. In the Bastrop soil, the degradation rate for the acid, base, and neutral fractions were 94, 0, and 82%, respectively. Thus, for the COMBO waste, the most rapid rate of degradation occurred in the acid fraction and the least rapid rate in the base fraction. In addition, the average total degradation rate for the COMBO waste in both soils was 49% from day 0 to 180 and 60% from day 180 to 360.

A comparison of the average degradation rates of the three wastes indicates that at day 360, the COMBO waste was 79% degraded, while the PENT S and SWRI wastes were degraded 65 and 57%, respectively. The average total rates of degradation for day 0 to 180 and day 180 to 360 were 57 and 18% for the PENT S waste, 24 and 44% for the SWRI waste, and 49 and 60% for the COMBO waste, respectively. For the COMBO and SWRI wastes, the most rapid rate of degradation occurred in the Bastrop soil; whereas, the PENT S waste degraded most rapidly in the Norwood soil (Figure 82). Previous studies with two API-separator sludges found the rate of degradation in the Norwood soil to be from 30 to 60% greater than the rate of degradation in the Bastrop soil (Brown and Donnelly, 1983). The half-life of all three wastes, as measured by residual hydrocarbon content, was less than 360 days. Thus, these preliminary results indicate that all three wastes were transformed and degraded by soil incorporation.

The evaluation of residual hydrocarbon data does not, however, provide an indication of the affect of degradation on the mutagenic activity of waste constituents. Only through the use of bioassays can these parameters be measured. One of the major limitations of using these bioassays is the absence of a standard procedure for the statistical evaluation of environmental data. While there have been several procedures suggested for the analysis of bioassay data (Commoner, 1976; Munson and Goodhead, 1979; Horn et al., 1983; Chu et al., 1981; Katz, 1979; Bernstein et al., 1982; and Moore and Felton, 1983), none of these procedures include adjustments for background levels of mutagenic activity in environmental samples. The bioassay data from the greenhouse study were evaluated using two procedures. Comparisons that were made on a weight basis reviewed the total mutation frequency at five equally spaced dose levels. Data was accumulated on each sampling date for all three fractions of each waste from three boxes of each soil. Thus, each value in Tables 67 to 90 and Figures 83 through 106 represents the mean of 12 plates from at least two independent experiments. This data is an expression of the mutagenic potential of the residual hydrocarbons in the soil and does not account for losses due to degradation. However, an indication of the effect of degradation on the mutagenic potential of the residual hydrocarbons in the soil is provided by the data. Comparisons that were made on a volume basis reviewed the mutagenic activity ratio of a constant volume of soil and can be used to determine the reduction of the hazardous

TABLE 66. TOTAL HYDROCARBONS EXTRACTED FROM SOIL AMENDED WITH COMBINED API SEPARATOR SLOP-OIL EMULSION SOLID

	Extractable Hydrocarbons (mg/g)				% Degraded
	Days After Appplication				
Sample	0	90	180	360	Day 360
Norwood Soil					
Acid	1.5	-	1.3 ± 0.1	0.33	78
Base	0.4	-	0.4 ± 0.1	0.1 ± .09	75
Neutral	21.1	-	17.7 ± 2.3	5.5 ± 0.4	74
Total	23.0	-	19.3 ± 2.3	6.0 ± 0.45	74
Bastrop Soil					
Acid	3.6	-	0.8 ± 0.2	0.2 ± .05	94
Base	0.7	-	0.2 ± 0.1	1.0 ± .02	0
Neutral	29.7	-	8.7 ± 0.9	5.3 ± 1.2	82
Total	34.0	-	9.7 ± 0.6	5.7 ± 1.3	83

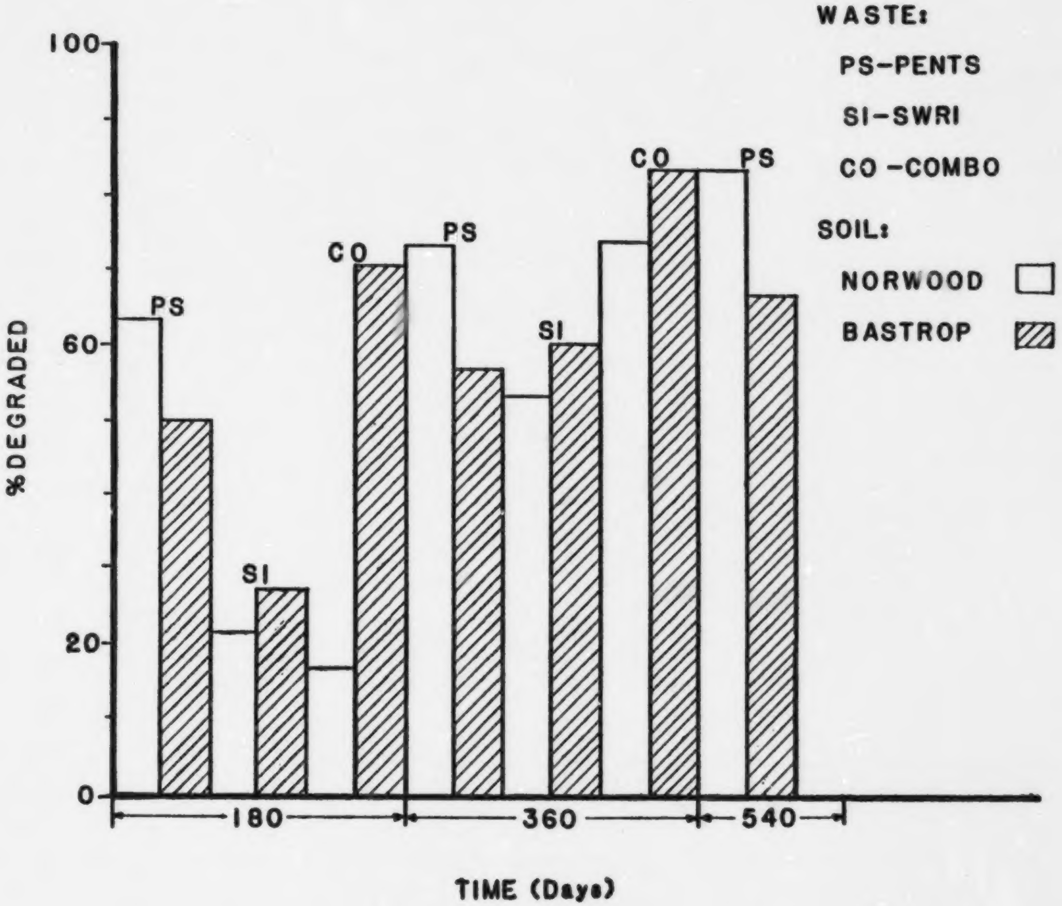


Figure 82. Degradation rate of total extractable hydrocarbons in Norwood and Bastrop soils amended with PENT S (PS), SWRI (SI), and Combo (CO) waste.

waste characteristics by land application. Comparisons made on a volume basis account for changes in the hydrocarbon content of the soil over time, as well as variations in the biological test system.

To determine mutagenicity on a volume basis, the mutagenic activity ratio (Commoner, 1976) is first calculated for the four highest non-toxic dose levels. The mutagenic activity ratio is calculated using the equation:

$$MAR = \frac{C_n - C_o}{C_A}$$

where C_n is the total number of revertant colonies on experimental plate at dose n (average of four plates); C_o is the number of revertant colonies on solvent control plates the same day the test was run using the same strain and tissue preparation (average of eight plates); and C_A is the historical average of solvent control plates for the year the test was run. Once the MAR's were calculated for each set of data, the mutagenic activity ratio for a specific volume of soil was calculated using the equation:

$$R_v = [\frac{V}{W_T}][MAR]$$

where V is the volume of soil (gram equivalents); W_T is the volume of soil equivalent to dose of residue = DOSE (mg/plate) - extractable hydrocarbons (mg/g); and MAR is the mutagenic activity ratio. The mutagenic activity ratios which are used calculate the R_V were calculated from the two non-toxic dose levels closest to the selected volume of soil which fell on the linear portion of the dose-response curve. The selected volume of soil was that volume of soil which fell between two dose levels which were used in the bioassays and was comparable to the optimum dose for the volume of soil extracted on Day 0. The extracts of the three fractions from each soil-waste mixture were tested at five equally spaced dose levels ranging from .01 to 1.0 mg/plate. For the acid and base fractions, these dose levels are equivalent to the extract from approximately .05 to 5.0 g of soil. The neutral fraction, which accounted for approximately 90% of the residual hydrocarbons in the soil-waste mixtures, was tested at the same dose levels on the basis of residual hydrocarbons that were equivalent to the extract from approximately 0.2 to 20 milligrams of soil. Thus, in order to calculate R_V using MAR's derived from the linear portion of the dose-response curve, the volume of soil used for comparing the neutral fraction will be one tenth the volume used for the acid and base fractions. By employing these calculations, the mutagenic potential of equal volumes or masses of soil can be compared. In addition, these calculations will account for the variability of the bioassay and the reduction in the amount of extractable hydrocarbon in the soil over time. The data from the Salmonella assay was evaluated on both a weight and volume basis, while the data from the Aspergillus assay was only evaluated on a volume basis.

The dose-response curves with strains TA98 and TA100 for the acid, base, and neutral fractions of the PENT S waste amended Norwood soil are provided in Figures 83 through 88 and Tables 67 through 72. In the acid fraction, the maximum response in strain TA98 was induced by the sample collected on day 180 (Figure 83 and Table 67); while, in strain TA100 the maximum response was induced by the sample collected on day 360 (Figure 86 and Table 70). In both strains, the response induced by the sample collected on day 540 was appreciably lower than the maximum response induced by samples collected prior to day 540. A similar trend was observed in the base fraction from PENT S waste amended Norwood soil. For strain TA98, the maximum response observed in the base fraction was induced by the sample collected on day 360 (Figure 84 and Table 68), while in strain TA100 the sample collected on day 540 induced the maximum response (Figure 87 and Table 71). In addition, significant levels of direct-acting mutagens were detected on day 540, whereas no direct-acting mutagens were detected in the waste or soil samples collected prior to this date. The maximum response induced by the neutral fraction was observed in the sample collected on day 180 in strain TA98 (Figure 85 and Table 69) and in the sample collected on day 360 in TA100 (Figure 88 and Table 72). These results indicate that for the PENT S waste amended Norwood soil, the factors affecting the mutagenic potential of the residual hydrocarbons initially increased but eventually decreased the mutagenicity of the acid and neutral fractions. While in the basic fraction, the mutagenicity was increased over the entire 540 day period with an apparent increase in direct-acting mutagenicity also occurring.

In the PENT S waste amended Bastrop soil, the overall rate of hydrocarbon degradation and the rate of detoxification appeared to be lower than the rate in the Norwood soil. The maximum mutagenic response induced by the acid, base, and neutral fractions was observed in the sample collected on day 540 in strain TA98 (Figures 89, 90, and 91, and Tables 73, 74, and 75) and on day 360 in strain TA100 (Figures 92, 93, and 94 and Tables 76, 77, and 78). As occurred in the Norwood soil, direct-acting mutagens were detected in the base fraction collected on day 540 in both strains (Figures 90 and 93 and Tables 74 and 77). Thus, the mutagenic activity of all three fractions from the PENT S waste amended Bastrop soil appeared to reach a maximum on day 360 as measured with strain TA100, while the maximum response in strain TA98 was induced by the sample collected on day 540.

The dose-response curves for the hydrocarbons extracted from the SWRI waste amended Norwood soil are given in Figures 95, 96, and 97. The mutagenic activity of the acid fraction increased from day 0 to 180 and from day 180 to 360 (Figure 95 and Table 79). While the mutagenic potential of the acid fraction of the soil-waste mixture from days 0 and 180 were less than the mutagenic potential of the acid fraction of the

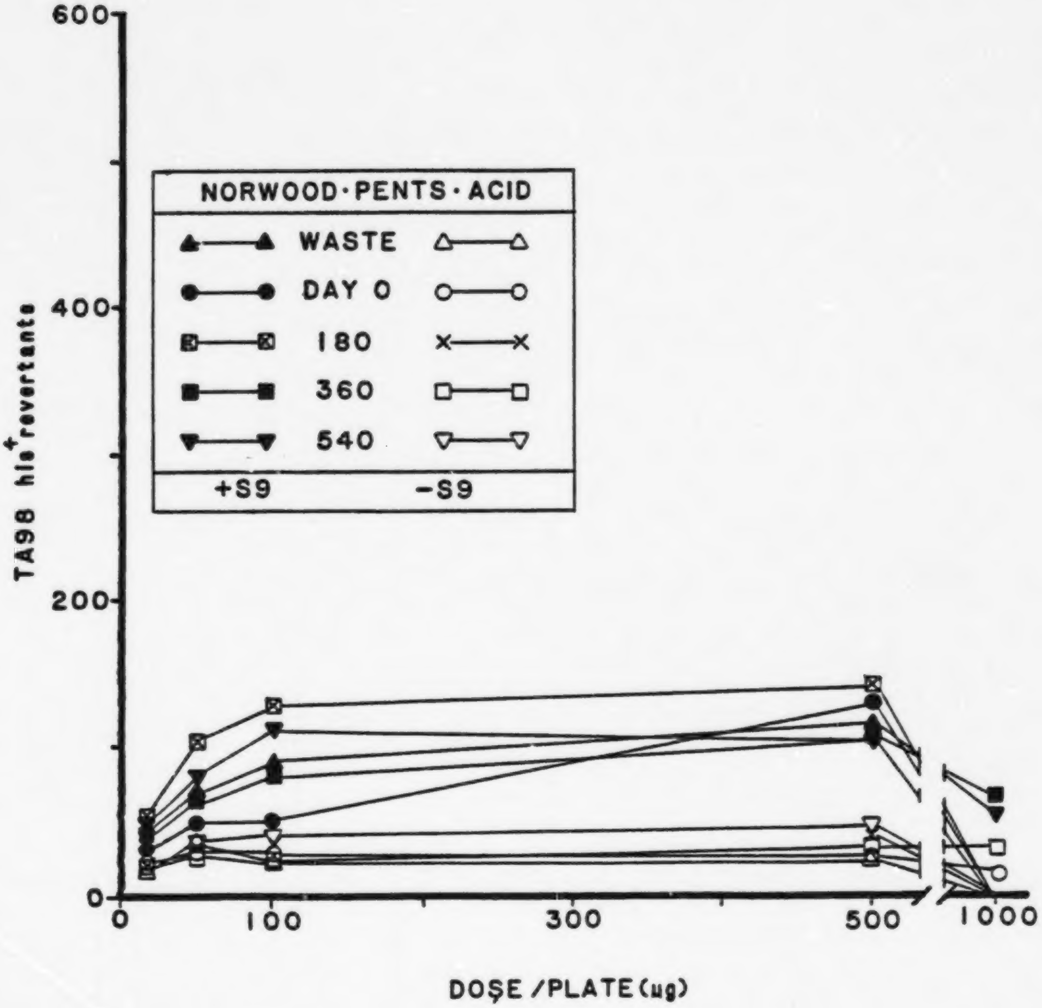


Figure 83. Mutagenic activity of acid fraction of PENT S amended Norwood soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

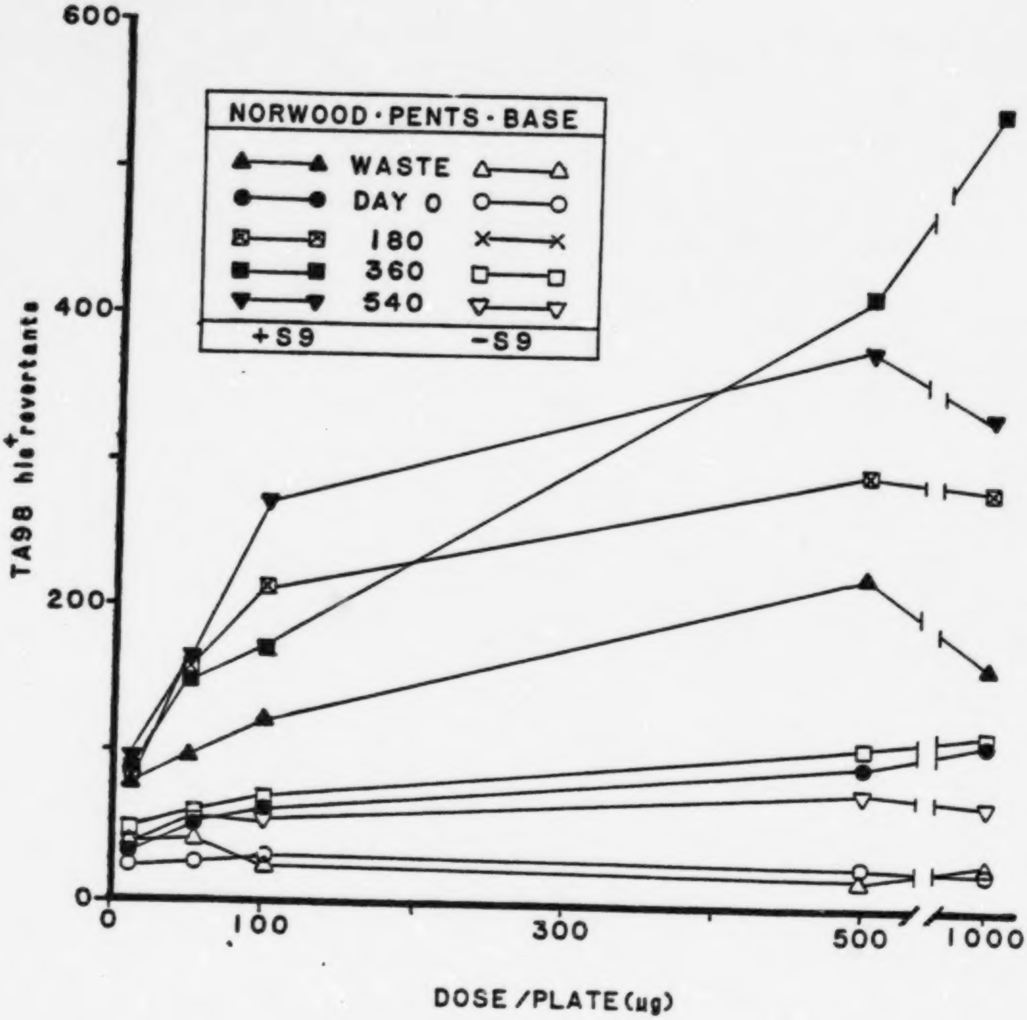


Figure 84. Mutagenic activity of base fraction of PENT S amended Norwood soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

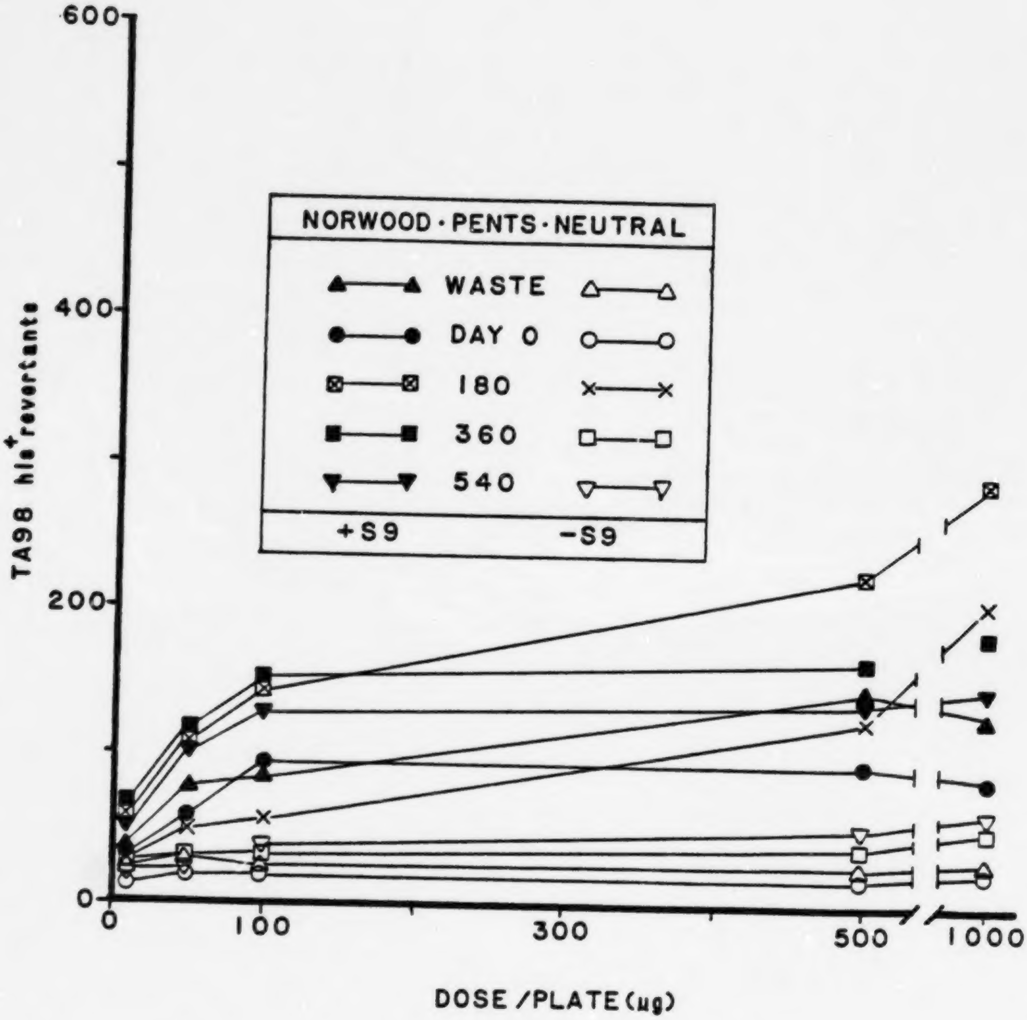


Figure 85. Mutagenic activity of neutral fraction of PENT S amended Norwood soil as measured with S typhimurium strain TA98 with and without metabolic activation.

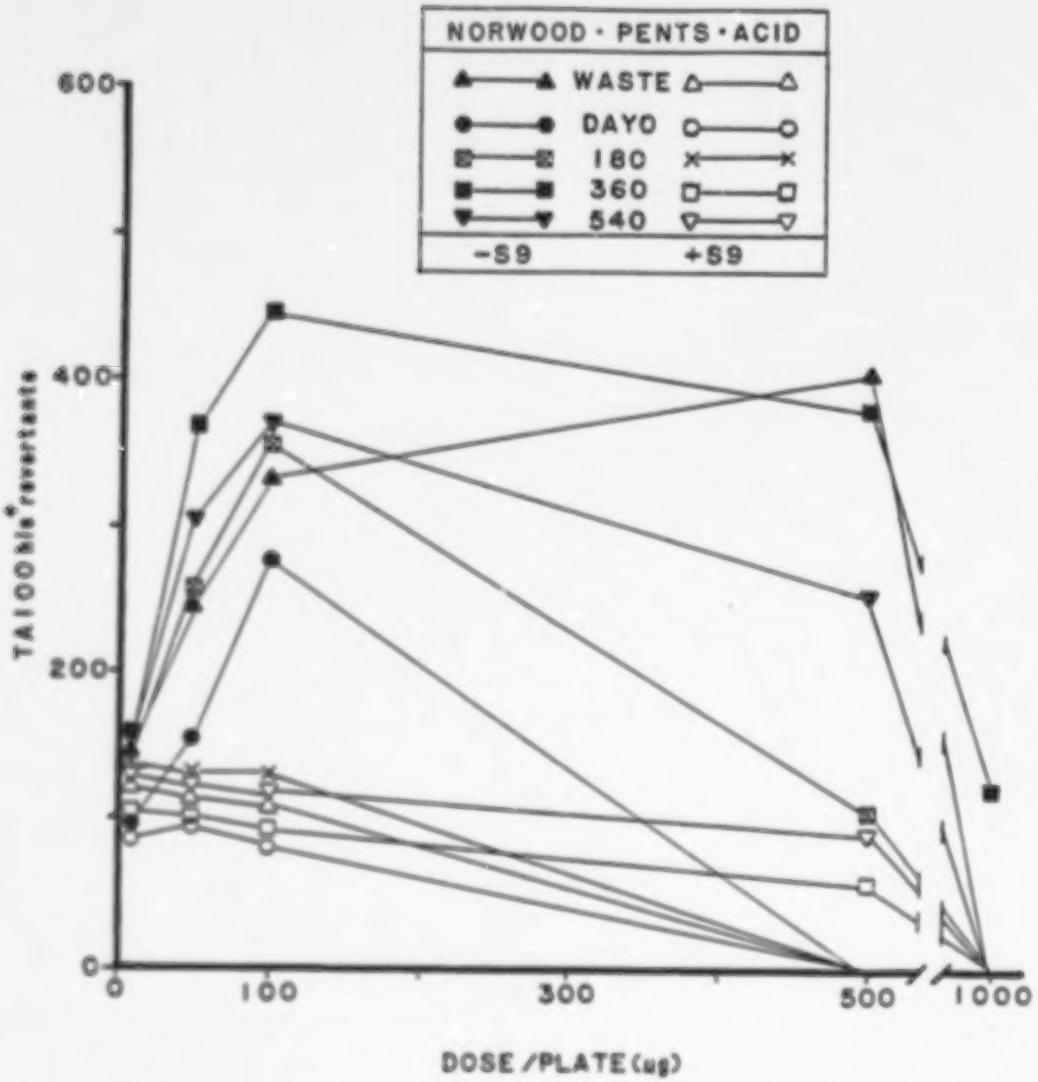


Figure 86. Mutagenic activity of acid fraction of PENT S amended Norwood soil as measured with S typhimurium strain TA100 with and without metabolic activation.

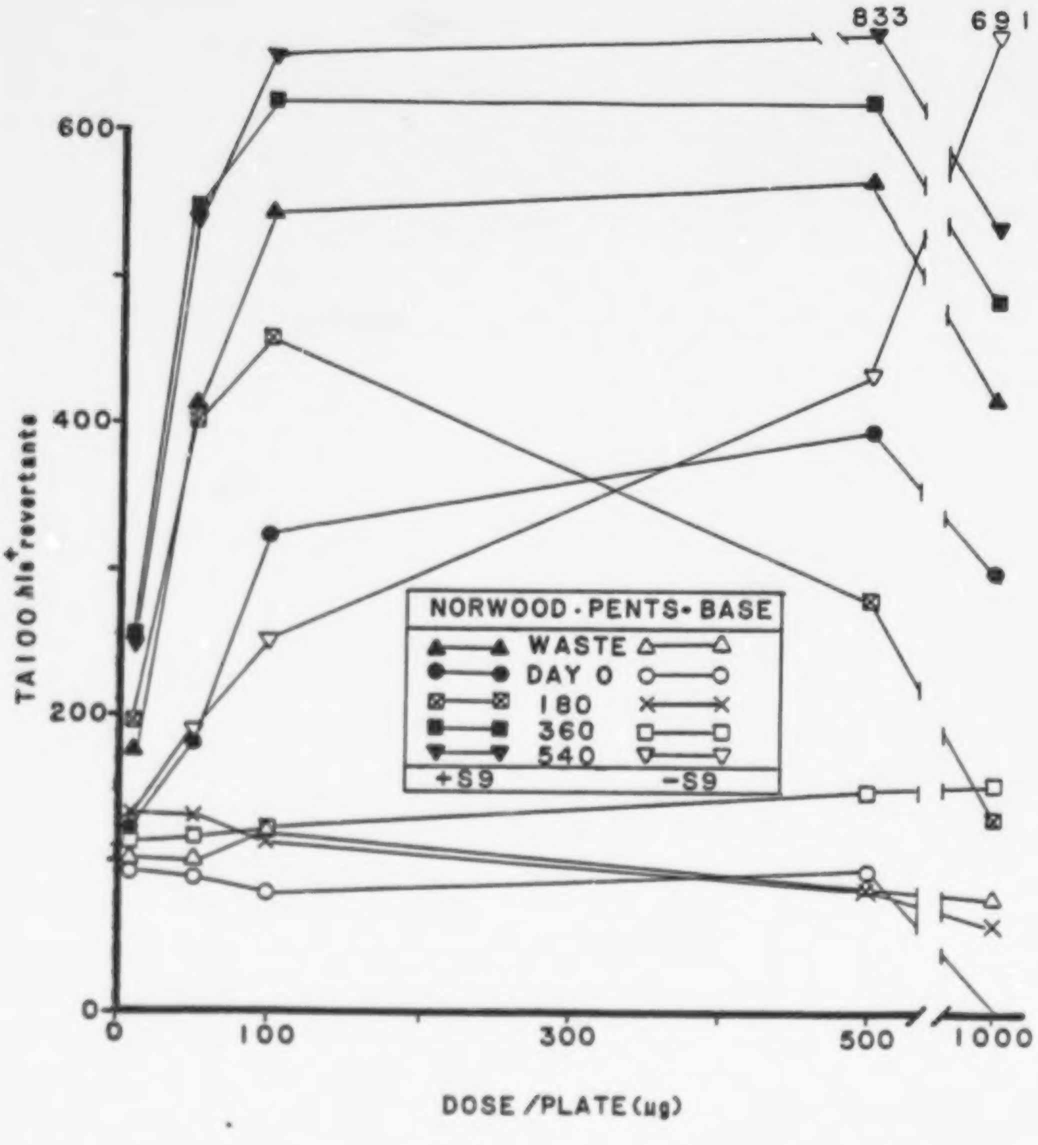


Figure 87. Mutagenic activity of base fraction of PENT S amended Norwood soil as measured with S typhimurium strain TA100 with and without metabolic activation.

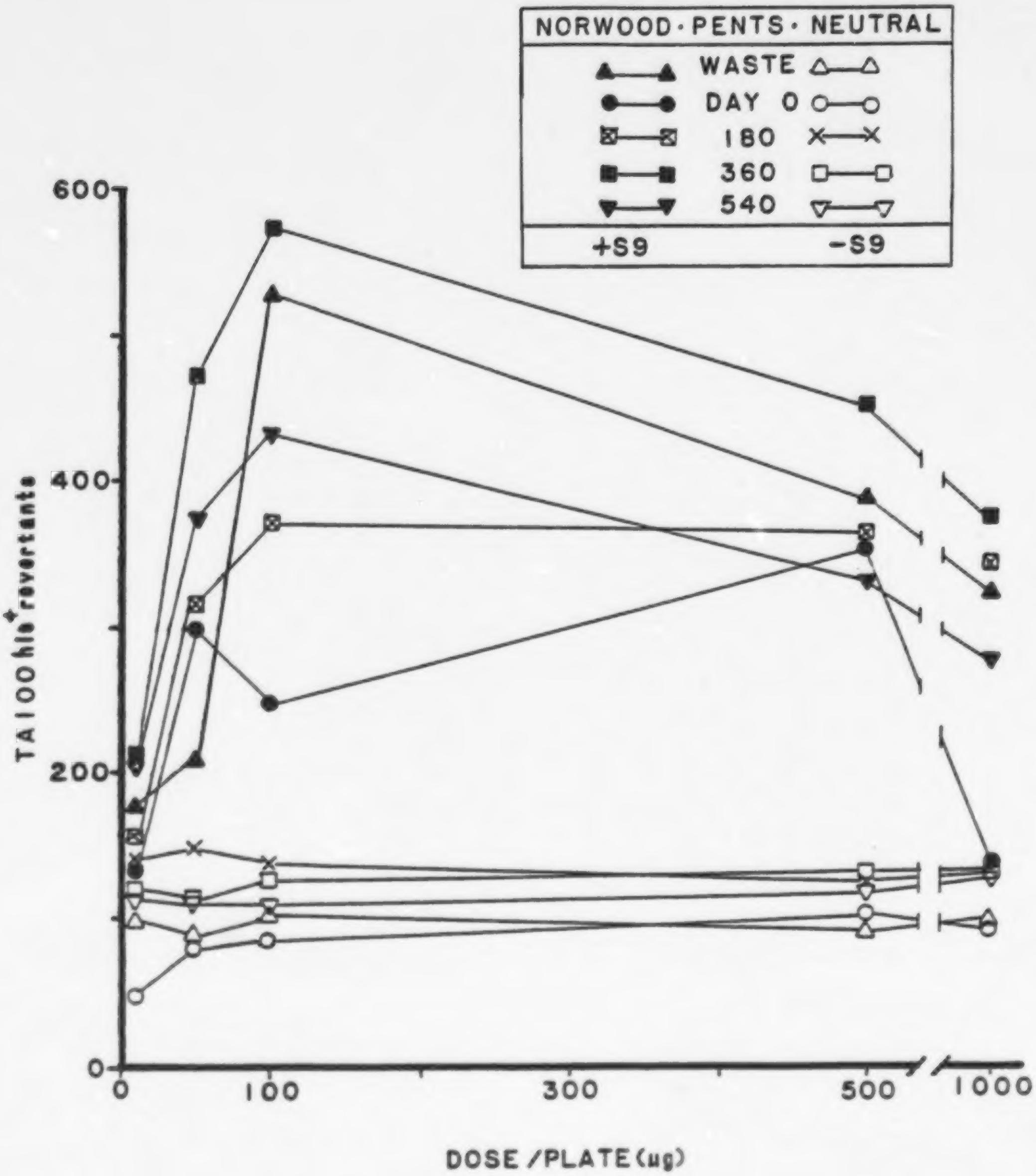


Figure 88. Mutagenic activity of neutral fraction of PENT S amended Norwood soil as measured with S. typhimurium strain TA100 with and without metabolic activation.

TABLE 67. MUTAGENIC ACTIVITY OF ACID FRACTION OF PENT S WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	21 ± 2.5	Toxic	20 ± 8.4	21 ± 5.0	21 ± 5.6	17 ± 2.6
	+	22 ± 4.4	Toxic	126 ± 11.3	49 ± 5.9	42 ± 17.7	17 ± 6.2
Day 180	-	27 ± 2.6	Toxic	34 ± 18.8	40 ± 5.7	37 ± 6.2	33 ± 4.4
	+	36 ± 2.9	Toxic	144 ± 60.6	120 ± 25.3	100 ± 37.2	59 ± 11.3
Day 360	-	28 ± 0.2	Toxic	37 ± 5.3	42 ± 21.8	41 ± 25.5	42 ± 27.3
	+	37 ± 0.2	85 ± 35.0	157 ± 18.9	133 ± 6.9	112 ± 10.5	68 ± 18.9
Day 540	-	28 ± 0.8	21 ± 8.4	37 ± 6.4	32 ± 7.3	35 ± 10.7	23 ± 1.1
	+	38 ± 3.3	44 ± 19.8	110 ± 12.9	111 ± 27.9	72 ± 23.9	43 ± 5.3

TABLE 68. MUTAGENIC ACTIVITY OF BASE FRACTION OF PENT S WASTE AMENDED NORWOOD SOIL AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	21 ± 2.5	24 ± 6.3	21 ± 4.2	24 ± 3.6	20 ± 7.8	19 ± 3.3
	+	22 ± 4.4	112 ± 12.4	98 ± 31.3	62 ± 20.4	37 ± 7.5	22 ± 7.0
Day 180	-	27 ± 2.6	60 ± 32.9	51 ± 34.8	38 ± 9.7	35 ± 7.8	31 ± 6.7
	+	36 ± 2.9	284 ±137.8	293 ± 115.0	215 ± 53.0	157 ± 16.5	72 ± 25.8
Day 360	-	29 ± 0.9	111 ± 15.4	106 ± 16.7	62 ± 22.8	55 ± 26.8	44 ± 28.5
	+	39 ± 5.0	540 ± 23.7	417 ± 22.4	170 ± 14.4	151 ± 34.6	75 ± 11.5
Day 540	-	27 ± 0.8	59 ± 11.0	74 ± 40.0	52 ± 28.0	34 ± 2.8	28 ± .71
	+	40 ± 3.3	334 ±158.0	381 ± 133.0	274 ± 147.0	163 ± 29.0	68 ± 13.0

TABLE 69. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF PENT S WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	21 ± 3.6	22 ± 6.7	19 ± 5.1	24 ± 3.6	20 ± 7.8	19 ± 3.3
	+	21 ± 1.8	86 ± 7.6	96 ± 28.2	62 ± 20.4	37 ± 7.5	22 ± 7.0
Day 180	-	26 ± 2.4	204 ± 154.5	126 ± 68.3	38 ± 9.7	35 ± 7.8	31 ± 6.7
	+	36 ± 2.9	281 ± 78.3	220 ± 40.7	215 ± 53.0	157 ± 16.5	72 ± 25.8
Day 360	-	28 ± 1.0	50 ± 5.5	36 ± 1.4	62 ± 22.8	55 ± 26.8	44 ± 28.5
	+	36 ± 1.0	184 ± 24.1	160 ± 16.9	144 ± 20.5	119 ± 10.7	64 ± 9.2
Day 540	-	27 ± 0.8	50 ± 11.5	41 ± 5.9	31 ± 4.1	27 ± 1.8	24 ± 1.1
	+	39 ± 2.9	144 ± 44.7	136 ± 22.6	128 ± 7.7	105 ± 10.3	57 ± 9.8

TABLE 70. MUTAGENIC ACTIVITY OF ACID FRACTION OF PENT S WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA100 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA100 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	93 ± 18.9	Toxic	Toxic	84 ± 7.4	87 ± 25.4	85 ± 11.0
	+	40 ± 11.9	Toxic	226 ± 17.3	202 ± 16.7	162 ± 35.4	118 ± 29.2
Day 180	-	105 ± 5.1	Toxic	Toxic	94 ± 22.4	105 ± 13.7	101 ± 14.9
	+	107 ± 17.8	Toxic	307*	362 ±116.9	275 ± 61.6	165 ± 5.7
Day 360	-	156 ± 6.0	Toxic	Toxic	147 ± 12.3	147 ± 8.8	139 ± 8.3
	+	135 ± 2.2	Toxic	287 ± 157.4	557 ± 88.9	419 ± 65.8	193 ± 9.6
Day 540	-	115 ± 7.5	Toxic	72 ± 11.0	113 ± 5.6	110 ± 4.7	116 ± 14.0
	+	126 ± 0.7	113 ± 25.8	235 ± 31.4	376 ± 28.1	288 ± 36.6	166 ± 17.7

* Mean represents average of only two samples; standard deviation not provided.

TABLE 71. MUTAGENIC ACTIVITY OF BASE FRACTION OF PENT S WASTE AMENDED NORWOOD SOIL AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA100 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA100 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	120 ± 21.9	Toxic	92 ± 11.3	80 ± 18.6	86 ± 8.8	95 ± 15.6
	+	134 ± 10.6	298 ± 22.0	395 ± 86.8	317 ± 37.1	181 ± 78.8	119 ± 27.03
Day 180	-	115 ± 13.1	56 ± 31.0	83 ± 15.0	113 ± 18.9	131 ± 25.6	132 ± 55.2
	+	118 ± 5.3	129 ± 52.7	277 ± 74.1	456 ±106.0	398 ± 80.8	193 ± 51.3
Day 360	-	138 ± 7.7	154 ± 10.5	148 ± 22.5	118 ± 29.1	114 ± 29.4	111 ± 30.7
	+	120 ± 1.7	486 ± 98.6	621 ± 108.8	618 ± 76.0	545 ± 65.4	259 ± 82.5
Day 540	-	115 ± 9.6	691 ± 90.9	433 ± 153.4	248 ± 68.8	187 ± 53.6	121 ± 6.8
	+	122 ± 3.4	528 ± 53.04	884 ± 63.9	652 ± 105.5	536 ± 23.0	259 ± 65.4

TABLE 72. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF PENT S WASTE AMENDED NORWOOD SOIL AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA100 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA100 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	120 ± 21.9	94 ± 13.0	105 ± 22.2	84 ± 17.0	77 ± 14.1	43 ± 47.9
	+	122 ± 40.1	139 ± 44.7	352 ± 45.6	244 ± 136.0	296 ± 41.1	133 ± 5.7
Day 180	-	111 ± 12.7	136 ± 39.1	125 ± 22.1	136 ± 29.5	148 ± 41.2	139 ± 38.6
	+	118 ± 5.3	346 ± 152.0	364 ± 124.0	372 ± 99.0	315 ± 59.2	156 ± 9.5
Day 360	-	130 ± 14.0	132 ± 28.9	126 ± 22.0	125 ± 23.5	112 ± 21.6	118 ± 14.8
	+	120 ± 1.7	375 ± 22.9	455 ± 23.2	571 ± 30.7	474 ± 13.3	211 ± 22.8
Day 540	-	120 ± 4.4	139 ± 9.0	122 ± 11.4	108 ± 9.4	111 ± 15.7	115 ± 3.6
	+	117 ± 8.1	280 ± 51.2	328 ± 40.8	434 ± 31.0	355 ± 14.6	201 ± 39.1

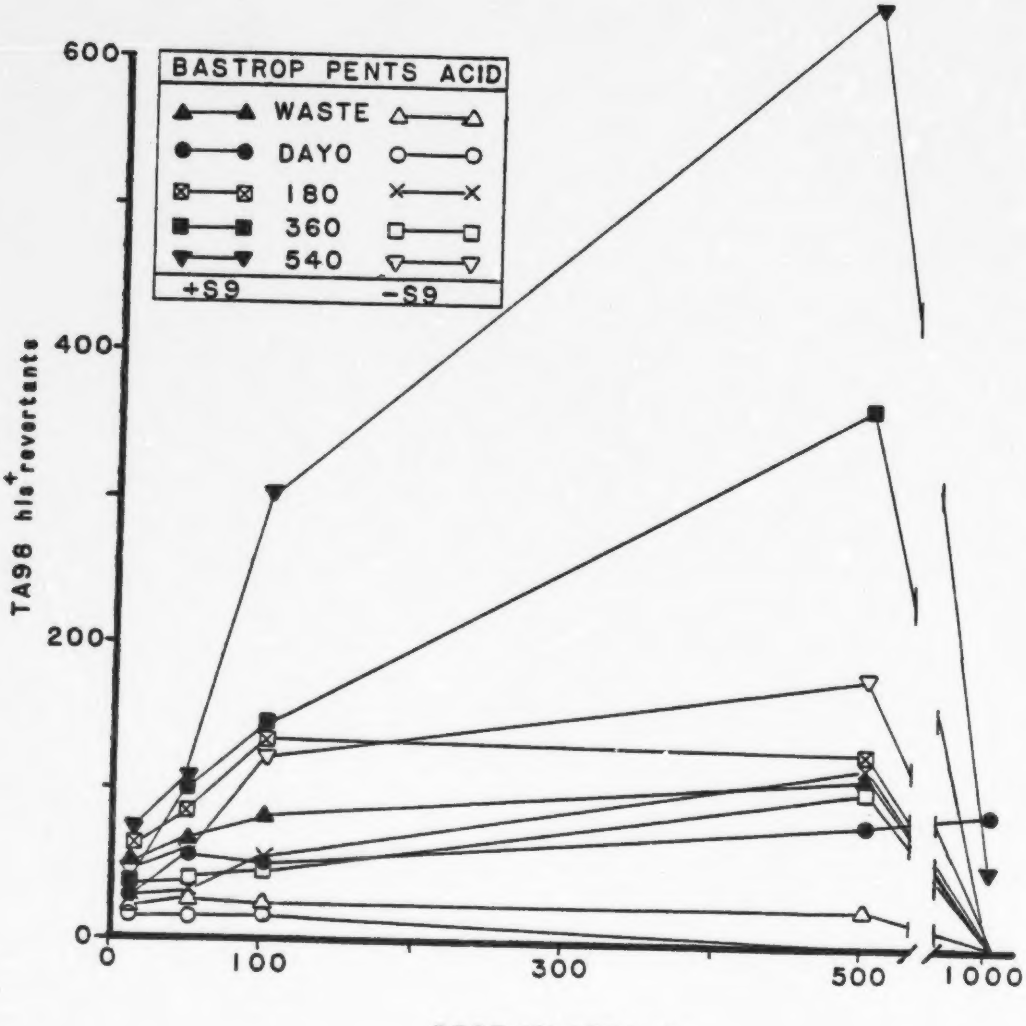


Figure 89. Mutagenic activity of acid fraction of PENT S amended Bastrop soil as measured with S typhimurium strain TA98 with and without metabolic activation.

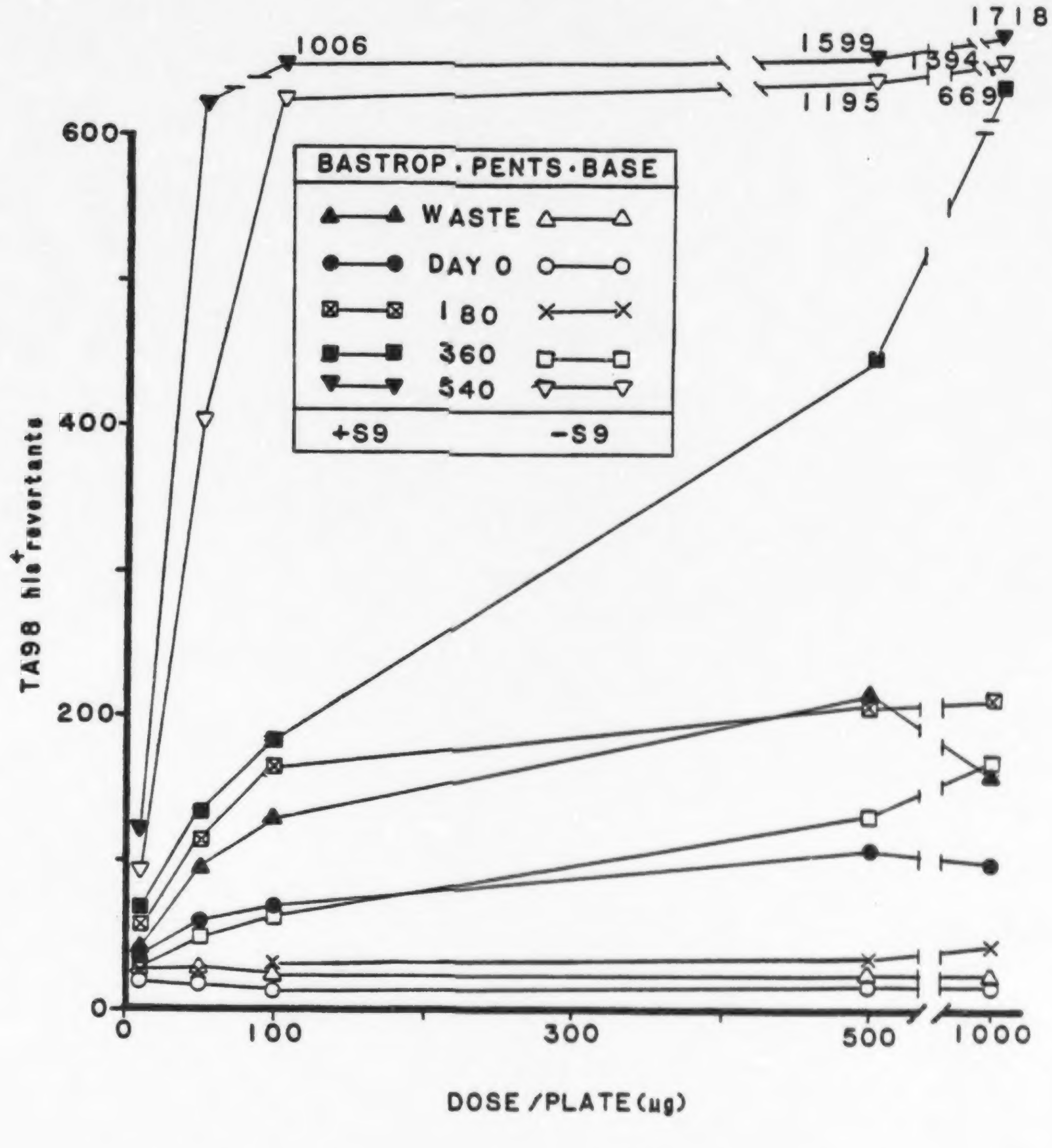


Figure 90. Mutagenic activity of base fraction of PENT S amended Bastrop soil as measured with S typhimurium strain TA98 with and without metabolic activation.

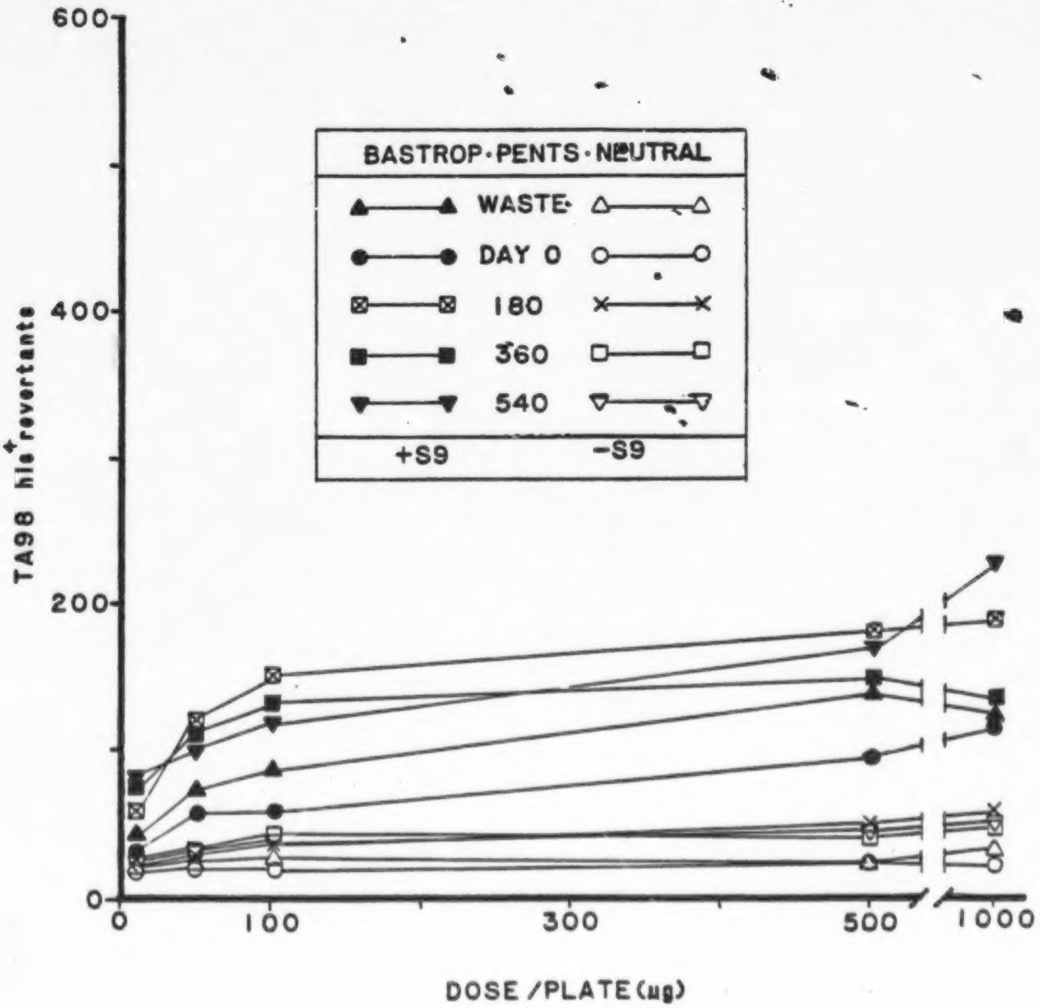


Figure 91. Mutagenic activity of neutral fraction of PENT S amended Bastrop soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

TABLE 73. MUTAGENIC ACTIVITY OF ACID FRACTION OF PENT S WASTE AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	21 ± 4.9	Toxic	Toxic	17 ± 5.1	16 ± 2.4	16 ± 1.8
	+	25 ± 4.3	87 ± 37.7	77 ± 10.5	53 ± 5.0	43 ± 4.6	27 ± 7.1
Day 180	-	28 ± 2.0	Toxic	117 ± 45.8	53 ± 3.8	39 ± 2.6	27 ± 3.0
	+	35 ± 3.7	Toxic	128 ± 41.2	138 ± 26.6	86 ± 9.0	58 ± 2.2
Day 360	-	26 ± 2.3	Toxic	110 ± 140.5	53 ± 42.0	37 ± 6.4	32 ± 5.1
	+	43 ± 3.3	Toxic	364 ± 432.0	139 ± 57.5	100 ± 23.4	56 ± 10.6
Day 540	-	31 ± 2.7	Toxic	184 ± 123.8	123 ± 8.0	60 ± 29.3	34 ± 8.3
	+	39 ± 5.7	513 ± 390.8	647 ± 421.5	304 ± 219.3	107 ± 29.9	59 ± 13.4

TABLE 74. MUTAGENIC ACTIVITY OF BASE FRACTION OF PENT S WASTE AMENDED BASTROP SOIL AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his^+ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	20 ± 1.9	17 ± 6.3	24 ± 4.7	15 ± 7.3	18 ± 2.5	19 ± 4.4
	+	25 ± 4.3	102 ± 24.4	109 ± 14.7	69 ± 25.25	60 ± 8.7	26 ± 2.4
Day 180	-	28 ± 2.0	42 ± 6.13	33 ± 3.5	31 ± 2.4	26 ± 4.9	23 ± 4.7
	+	35 ± 3.7	213 ± 48.0	208 ± 17.9	164 ± 14.0	116 ± 26.0	57 ± 10.3
Day 360	-	24 ± 3.2	167 ± 93.0	137 ± 70.0	60 ± 17.0	47 ± 11.0	30 ± 6.1
	+	43 ± 2.9	699 ± 372.7	452 ± 169.0	184 ± 9.5	132 ± 13.0	69 ± 11.0
Day 540	-	31 ± 2.7	1394 ± 1242.0	1195 ± 1140.0	624 ± 827.6	409 ± 536.0	99 ± 101.4
	+	39 ± 5.7	1718 ± 151.5	1600 ± 410.4	1006 ± 906.0	621 ± 671.0	119 ± 59.3

TABLE 75. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF PLOT 3 [illegible] AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his+ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	20 ± 1.8	23 ± 7.7	NT*	18 ± 4.3	17 ± 2.1	15 ± 3.2
	+	25 ± 4.3	122 ± 16.0	96 ± 16.6	56 ± 13.6	56 ± 20.1	27.5 ± 5.6
Day 180	-	27 ± 0.7	56 ± 21.7	50 ± 10.3	36 ± 3.0	33 ± 5.9	30 ± 4.0
	+	35 ± 3.7	109 ± 8.2	103 ± 7.0	149 ± 15.0	118 ± 30.5	57 ± 5.1
Day 360	-	23 ± 1.5	63 ± 8.6	36 ± 5.3	36 ± 4.9	27 ± 4.9	25 ± 2.5
	+	66 ± 3.6	137 ± 43.0	216 ± 17.0	132 ± 28.0	113 ± 41.0	68 ± 13.0
Day 540	-	31 ± 2.7	52 ± 12.0	38 ± 11.0	30 ± .71	30 ± 2.8	29 ± 1.6
	+	69 ± 5.7	226 ± 113.0	260 ± 47.0	131 ± 15.0	97 ± 10.6	66 ± 10.3

** Not tested due to limited amount of sample available.

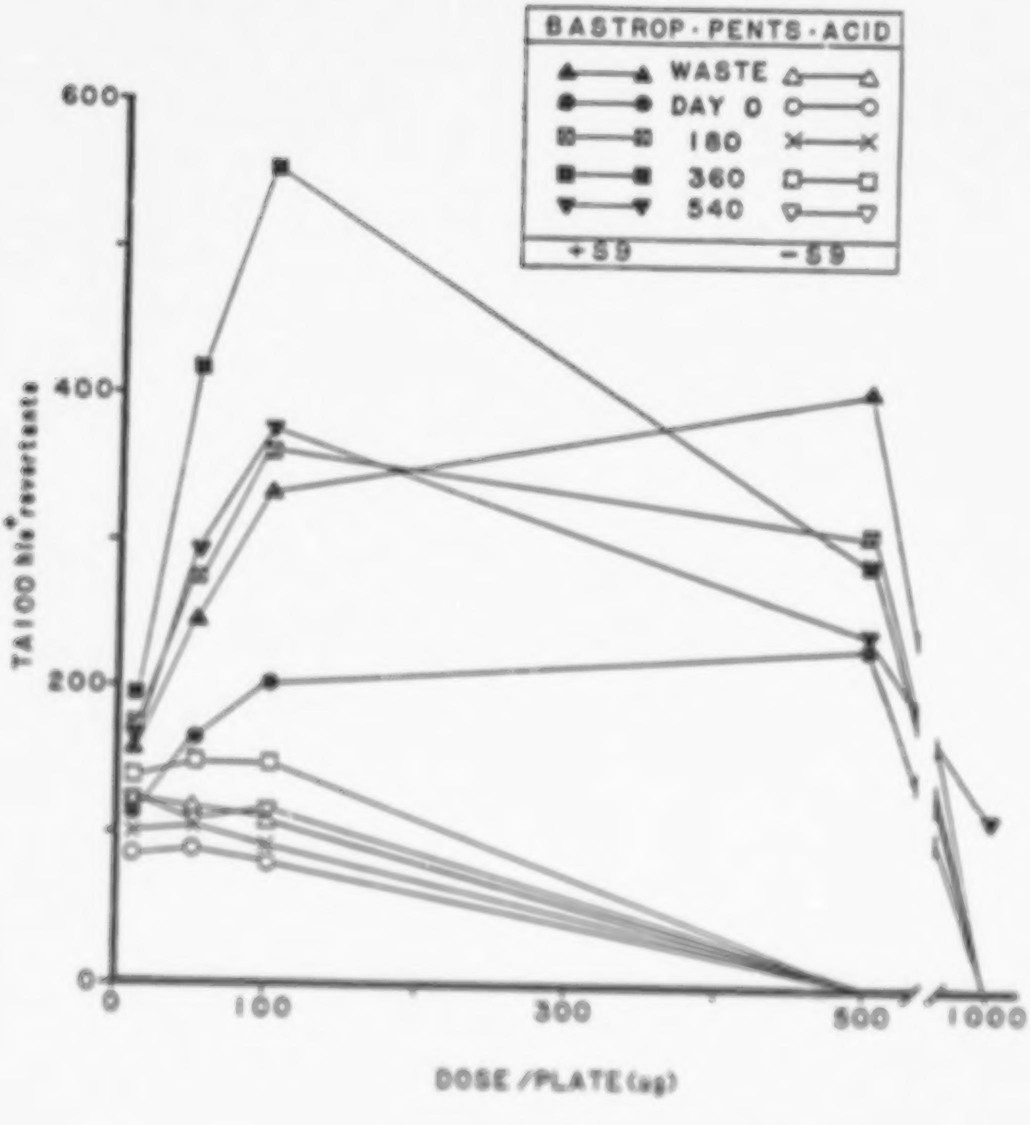


Figure 92. Mutagenic activity of acid fraction of PENT 5 amended Bastrop soil measured with _S typhimurium_ strain TA100 with and without metabolic activation.

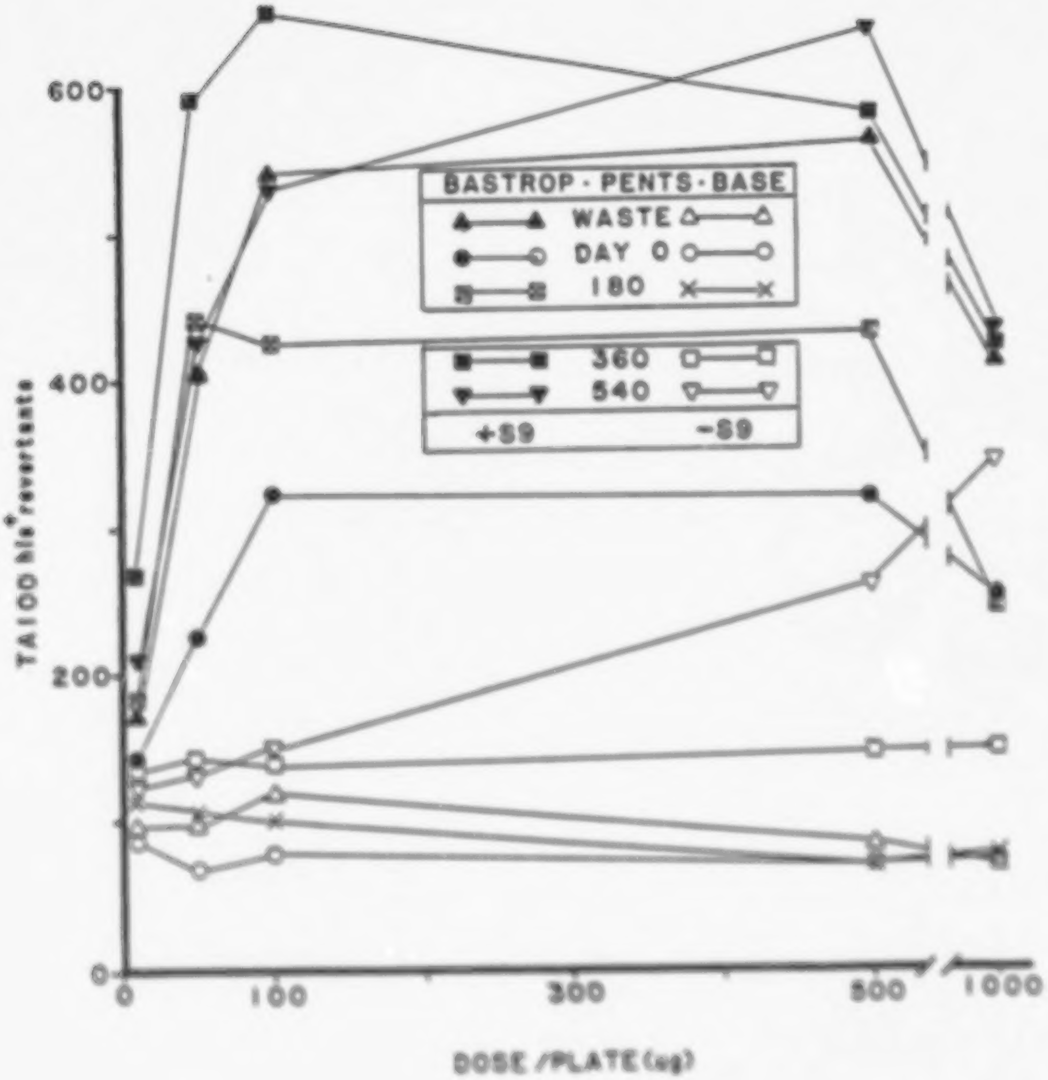


Figure 93. Mutagenic activity of base fraction of PENT S amended Bastrop soil as measured with *S typhimurium* strain TA100 with and without metabolic activation.

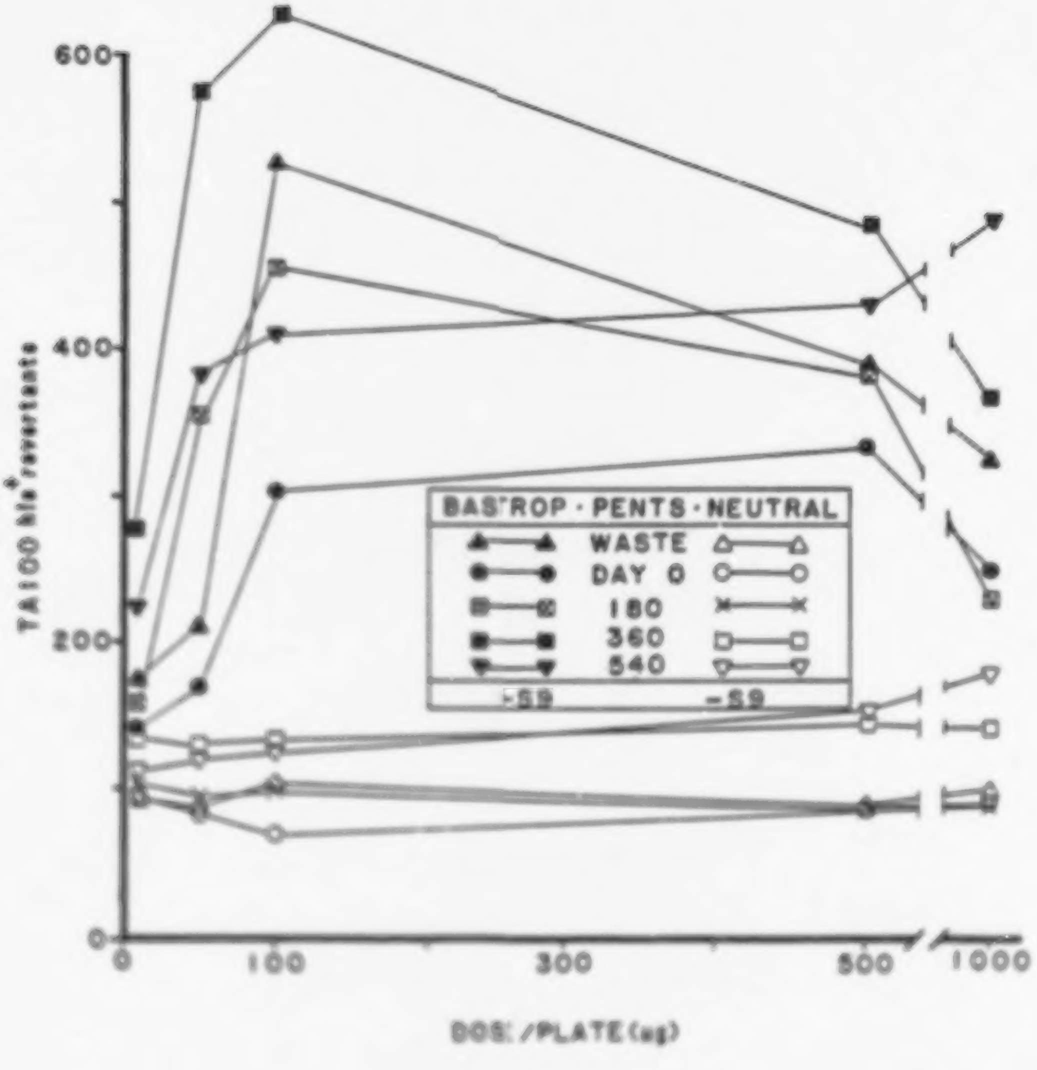


Figure 94. Mutagenic activity of neutral fractionof PENT 5 amended Bastrop soil asmeasured with S. typhimurium strain TA100 with and without metabolic activation.

TABLE 76. MUTAGENIC ACTIVITY OF ACID FRACTION OF PENT S WASTE AMENDED BASTROP SOIL AS MEASURED WITH *S. TYPHIMURIUM* STRAIN TA100 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA100 his$^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	120 ± 22.0	Toxic	Toxic	82 ± 11.0	97 ± 24.0	88 ± 11.0
	+	136 ± 11.0	Toxic	Toxic	278 ± 64.0	154 ± 35.7	100 ± 21.0
Day 180	-	115 ± 13.0	Toxic	Toxic	98 ± 2.2	127 ± 21.0	137 ± 31.0
	+	118 ± 5.0	Toxic	107 ± 9.2	358 ± 19.0	225 ± 34.0	137 ± 24.0
Day 360	-	127 ± 9.0	Toxic	61 ± 21.0	93 ± 21.0	97 ± 16.0	103 ± 29.0
	+	121 ± 1.9	Toxic	382 ± 121.9	448 ± 56.0	367 ± 46.0	169 ± 18.0
Day 540	-	121 ± 5.1	Toxic	90 ± 8.0	119 ± 5.8	121 ± 5.2	125 ± 4.3
	+	122 ± 0.7	121 ± 10.0	258 ± 74.6	369 ± 14.6	305 ± 57.0	158 ± 17.0

TABLE 77. MUTAGENIC ACTIVITY OF BASE FRACTION OF PENT 5 WASTE AMENDED BASTROP SOIL AS MEASURED WITH
S. TYPHIMURIUM STRAIN TA100 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA100 his + Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	85 ± 12.0	NT**	77 ± 11.6	789 ± 19.0	66 ± 7.1	84 ± 4.6
	+	135 ± 12.2	256 ± 37.8	321 ± 78.5	322 ± 30.7	226 ± 19.3	140 ± 28.6
Day 180	-	106 ± 5.1	73 ± 7.6	73 ± 7.6	103 ± 10.0	107 ± 7.7	115 ± 15.2
	+	107 ± 17.8	366 ± 39.3	436 ± 45.6	428 ± 138.0	450 ± 121.0	184 ± 10.2
Day 360	-	130 ± 10.0	149 ± 40.0	149 ± 2.1	142 ± 18.0	163 ± 15.0	139 ± 16.0
	+	123 ± 17.0	619 ± 146.0	588 ± 252.0	635 ± 245.0	591 ± 260.0	272 ± 118.0
Day 540	-	115 ± 7.5	365*	263*	150*	130*	121*
	+	127 ± 7.7	633*	663*	539*	427*	212*

* Mean represents average of only two samples; standard deviation not provided.
** Not tested due to limited amount of sample available.

TABLE [illegible]. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF PENT S WASTE AMENDED BASTROP SOIL AS MEASURED WITH
S. TYPHIMURIUM STRAIN TA100 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA100 his + Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	93 ± 11.6	92 ± 16.3	NT*	67 ± 7.8	NT	99 ± 13.1
	+	160 ± 12.9	310 ± 30.0	333 ± 39.1	299 ± 26.7	168 ± 46.4	142 ± 21.2
Day 180	-	105 ± 5.3	Toxic	Toxic	100 ± 12.6	93 ± 18.5	100 ± 8.4
	+	107 ± 17.8	230 ± 95.3	386 ± 19.6	456 ± 102.0	352 ± 79.5	158 ± 22.1
Day 360	-	137 ± 16.0	160 ± 19.0	161 ± 15.0	137 ± 16.0	132 ± 15.0	137 ± 18.0
	+	126 ± 17.0	368 ± 121.0	485 ± 139.0	620 ± 191.0	572 ±152.0	278 ± 93.0
Day 540	-	115 ± 0.0	178 ± 75.0	155 ± 49.6	128 ± 27.6	119 ± 16.9	114 ± 4.8
	+	126 ± 0.0	488 ± 325.0	426 ± 128.7	408 ± 73.9	380 ± 62.0	222 ± 23.2

* Not tested due to limited amount of sample available.

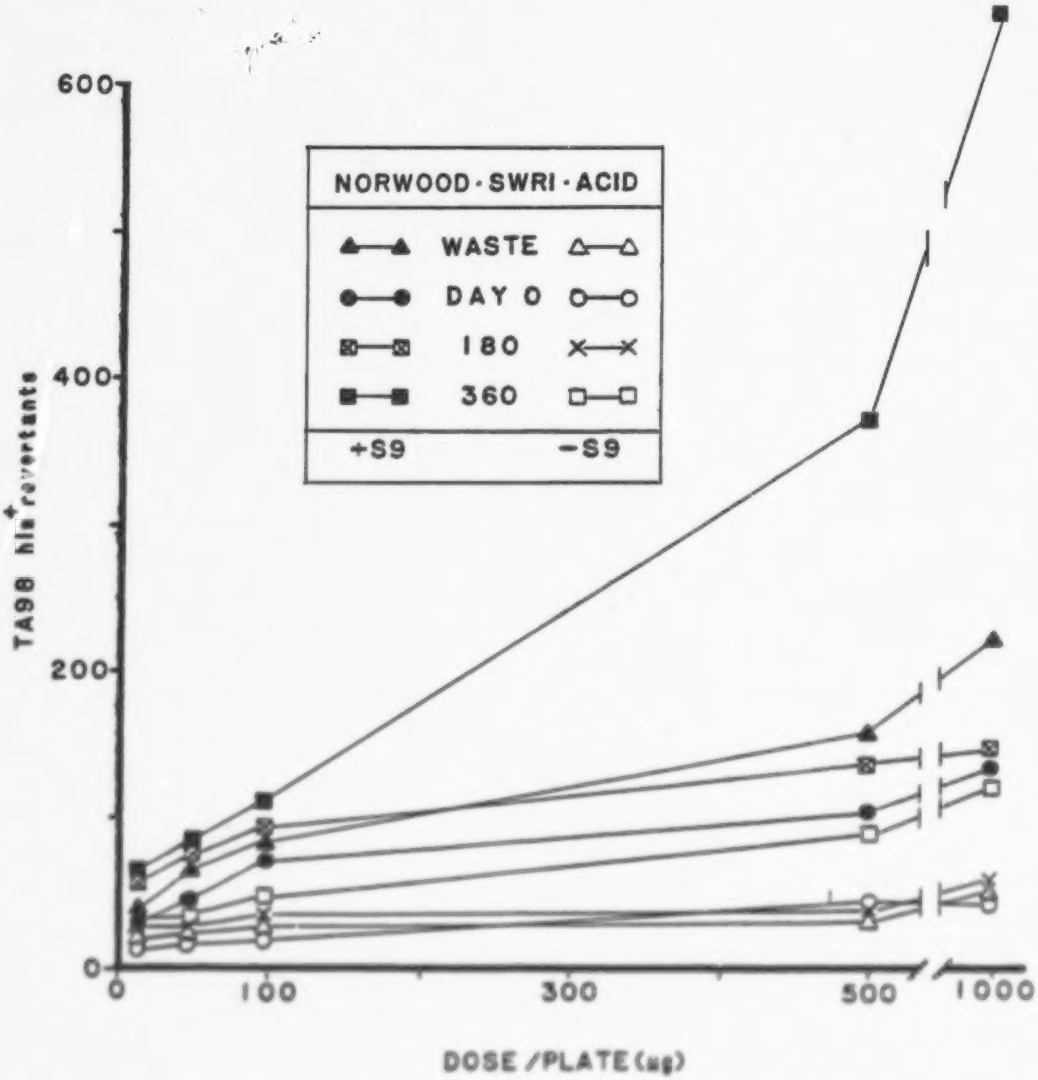


Figure 95. Mutagenic activity of acid fraction of SWRI amended Norwood soil as measured with S typhimurium strain TA98 with and without metabolic activation.

TABLE 79. MUTAGENIC ACTIVITY OF ACID FRACTION OF SWRI WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	24 ± 4.5	39 ± 19.4	40 ± 7.5	32 ± 7.2	26 ± 7.5	22 ± 7.3
	+	30 ± 5.0	138 ± 36.9	102 ± 9.0	77 ± 13.8	44 ± 22.4	34 ± 10.6
Day 180	-	25 ± 3.0	55 ± 2.6	37 ± 1.6	27 ± 3.1	21 ± 0.8	24 ± 0.8
	+	44 ± 8.6	139 ± 8.4	137 ± 4.4	86 ± 1.4	69 ± 13.3	46 ± 7.4
Day 360	-	27 ± 2.9	127 ± 123.0	92 ± 71.2	43 ± 30.6	34 ± 17.5	27 ± 9.6
	+	34 ± 6.5	650 ± 678.8	369 ± 336.2	113 ± 29.3	76 ± 26.5	58 ± 0.2

waste, the hydrocarbons in the acid fraction collected on day 360 induced a mutagenic response at higher dose levels that was two to three times greater than the response induced by the waste fraction. Similar results were observed in the base fraction with the maximum response induced by the sample collected on day 360. The base fraction from day 360 also induced a mutagenic response in the absence of metabolic activation, whereas neither the waste nor the base fraction of the samples collected prior to day 360 induced a mutagenic response without metabolic activation (Table 80 and Figure 96). In the neutral fraction of the SWRI waste amended Norwood soil, the maximum response observed in the study samples was induced by the hydrocarbons extracted from the waste (Figure 97). In addition, the mutagenic activity of the neutral fraction from day 360 was appreciably lower than both the waste and the day 0 sample (Table 81 and Figure 97). Since the neutral fraction accounts for greater than 90% of the total extractable hydrocarbon in the SWRI waste, the reduction in mutagenic activity observed in the neutral fraction after 360 days should compensate for increases observed in the acid and base fractions. Thus, the mutagenic potential of the total extractable hydrocarbons should be reduced 360 days following application of the SWRI waste to the Norwood soil.

For the Bastrop soil amended with the SWRI waste, the same general trend over time was observed (Tables 80, 81, and 82 and Figures 98, 99, and 100). The maximum response observed in both the acid and base fractions was induced by the sample collected on day 360 (Tables 82 and 83 and Figures 98 and 99). In the neutral fraction, the mutagenic activity of the residual hydrocarbons was virtually unchanged from day 0 through 360. At the highest dose level, the mutagenic activity of the residual hydrocarbons in the neutral fraction of the SWRI waste amended Bastrop soil from day 0, 180, and 360 was approximately one-half the level of activity induced by the hydrocarbons present in the neutral fraction of the waste (Table 84 and Figure 100). These results indicate that the mutagenic activity of total residual hydrocarbons in both the Norwood and the Bastrop soils collected 360 days after waste application were significantly less than the mutagenic activity of the hydrocarbons present in the waste.

The results from the biological analysis of the COMBO waste amended Norwood soil are presented in Tables 85, 86, and 87 and Figures 101, 102, and 103. The sample collected on day 360 induced the maximum response observed in the acid fraction from COMBO waste amended Norwood soil (Figure 101 and Table 85). In addition, direct-acting mutagens were detected in the acid fraction from day 360, whereas there were no direct-acting mutagens detected in the acid fraction of samples collected prior to day 360. In the base fraction, there was no appreciable difference in the mutagenic potential of the extract from the waste or the soil samples collected on day 0 and 180; however, the mutagenic potential of the base fraction from day 360 was approximately two times greater than the response induced by samples from previous dates. Without metabolic activation, the base fraction from day 360

TABLE 80. MUTAGENIC ACTIVITY OF BASE FRACTION OF SWRI WASTE AMENDED NORWOOD SOIL AS MEASURED WITH
S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his⁺ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
[illegible]	-	24 ± 4.5	30 ± 12.8	52 ± 5.9	31 ± 5.7	24 ± 7.8	19 ± 2.4
	+	30 ± 5.0	100 ± 33.6	103 ± 15.5	65 ± 6.3	61 ± 8.8	44 ± 10.2
Day 180	-	25 ± 3.3	59 ± 19.4	46 ± 16.5	43 ± 22.4	43 ± 35.5	36 ± 21.2
	+	45 ± 7.1	206 ± 23.9	149 ± 6.6	78 ± 9.4	66 ± 4.9	46 ± 4.5
Day 360	-	28 ± 2.6	171 ± 69.7	138 ± 49.9	54 ± 17.6	39 ± 14.9	25 ± 5.4
	+	33 ± 7.7	901 ± 409.4	400 ± 159.9	138 ± 30.8	80 ± 4.1	46 ± 8.6

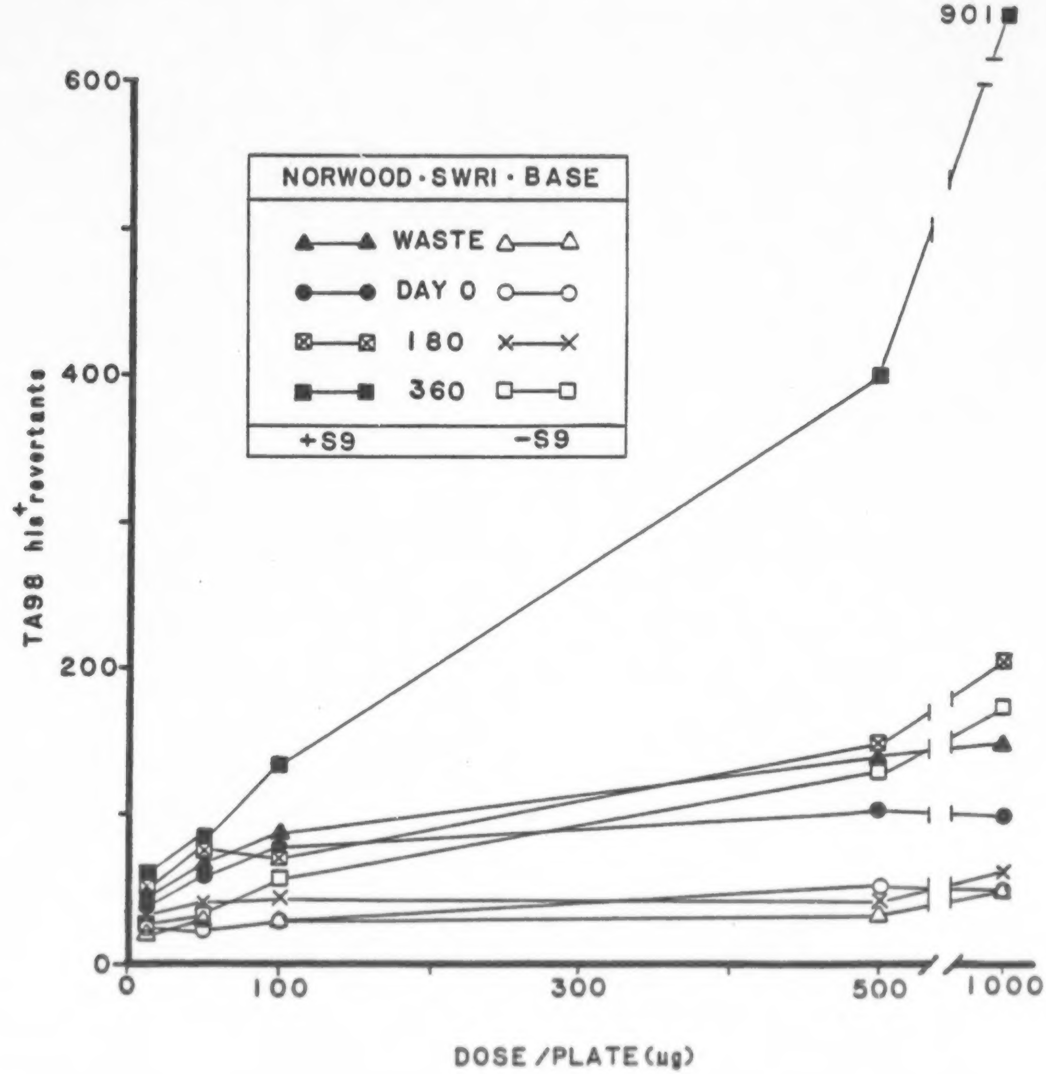


Figure 96. Mutagenic activity of base fraction of SWRI amended Norwood soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

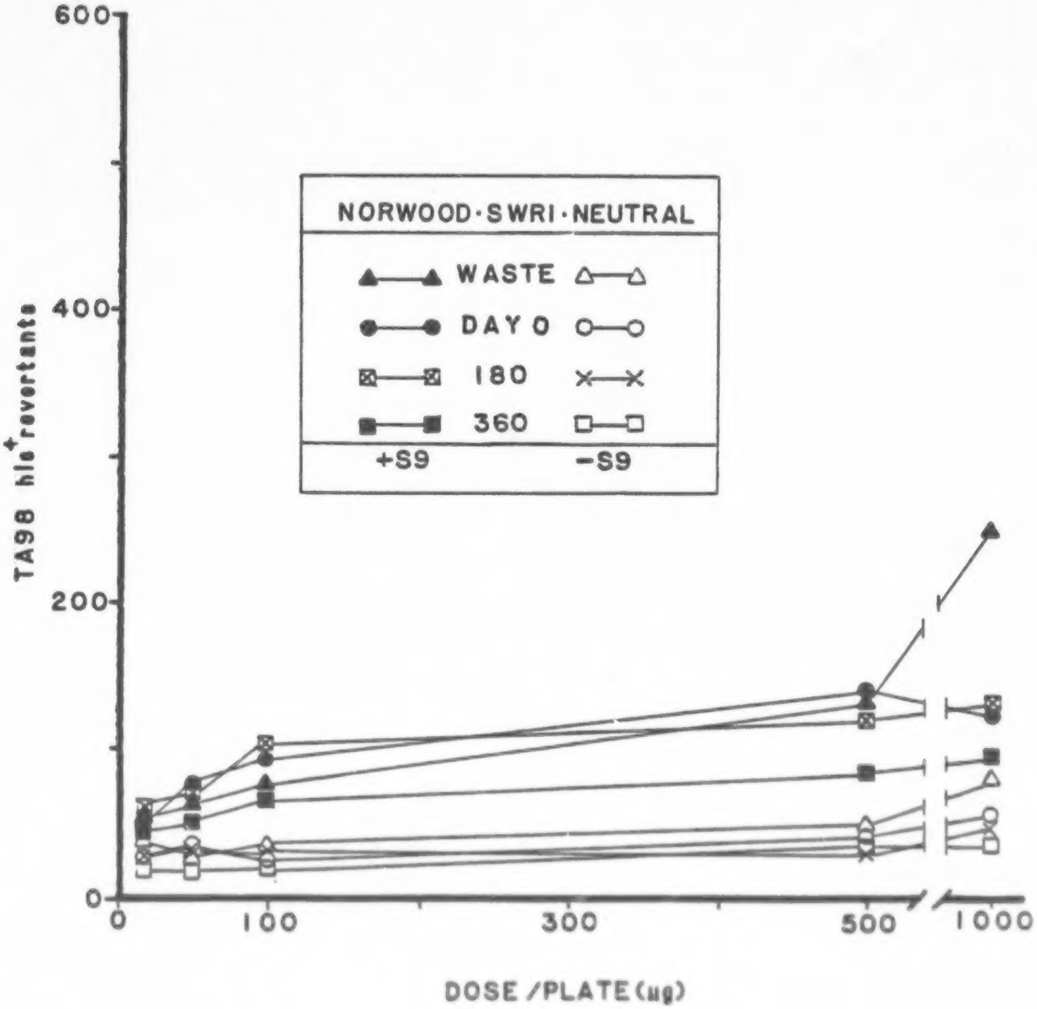


Figure 97. Mutagenic activity of neutral fraction of SWRI amended Norwood soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

TABLE 81. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF SWRI WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	23 ± 2.4	58 ± 12.8	42 ± 8.5	25 ± 6.5	30 ± 9.3	27 ± 2.2
	+	31 ± 5.5	125 ± 19.0	138 ± 7.8	90 ± 9.8	79 ± 13.3	50 ± 18.7
Day 180	-	35 ± 2.9	44 ± 5.7	30 ± 0.8	27 ± 3.2	23 ± 1.8	27 ± 2.3
	+	44 ± 1.2	127 ± 24.9	122 ± 16.6	95 ± 11.2	71 ± 11.9	53 ± 2.6
Day 360	-	26 ± 1.3	39 ± 8.4	31 ± 5.7	24 ± 3.1	24 ± 0.8	21 ± 1.9
	+	29 ± 2.0	92 ± 19.4	81 ± 4.9	66 ± 12.5	52 ± 11.4	38 ± 7.8

TABLE 82. MUTAGENIC ACTIVITY OF ACID FRACTION OF SWRI WASTE AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his^+ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	22 ± 3.4	156 ± 33.4	129 ± 26.7	39 ± 7.0	31 ± 7.3	26 ± 4.8
	+	32 ± 5.8	263 ± 23.7	188 ± 10.8	88 ± 20.3	63 ± 10.2	56 ± 11.9
Day 180	-	29 ± 8.3	52 ± 4.3	45 ± 9.2	32 ± 6.8	32 ± 2.3	29 ± 2.7
	+	43 ± 3.1	137 ± 30.1	139 ± 19.6	90 ± 14.2	76 ± 6.9	61 ± 8.0
Day 360	-	31 ± 4.7	86 ± 8.5	69 ± 8.3	40 ± 12.0	37 ± 4.2	30 ± 4.9
	+	40 ± 1.8	341 ± 151.0	198 ± 65.0	94 ± 9.0	42 ± 7.1	52 ± 5.7

TABLE 83. MUTAGENIC ACTIVITY OF BASE FRACTION OF SWRI WASTE AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	22 ± 3.4	NT*	70 ± 13.5	28 ± 6.4	26 ± 7.5	22 ± 5.3
	+	32 ± 5.8	134 ± 30.3	120 ± 40.9	71 ± 15.5	44 ± 22.4	39 ± 4.2
Day 180	-	29 ± 8.3	60 ± 32.9	50 ± 25.8	27 ± 4.1	21 ± 0.8	27 ± 3.9
	+	43 ± 3.0	243 ± 131.9	192 ± 68.7	93 ± 14.1	69 ± 13.3	55 ± 5.0
Day 360	-	31 ± 4.7	150 ± 101.3	84 ± 11.8	38 ± 10.3	28 ± 1.75	26 ± 4.2
	+	40 ± 1.8	494 ± 291.0	319 ± 158.8	89 ± 16.0	73 ± 6.4	45 ± 6.5

* Not tested due to limited amount of sample available.

TABLE 84. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF SWRI WASTE AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	23 ± 2.8	35 ± 5.6	36 ± 4.0	23 ± 4.9	28 ± 3.3	22 ± 4.9
	+	28 ± 9.2	117 ± 26.4	107 ± 7.0	63 ± 9.9	41 ± 9.5	30 ± 8.1
Day 180	-	29 ± 8.3	56 ± 35.7	45 ± 19.0	32 ± 4.9	28 ± 4.1	24 ± 7.1
	+	43 ± 3.1	142 ± 7.8	130 ± 15.6	87 ± 7.5	69 ± 8.0	55 ± 1.6
Day 360	-	31 ± 4.4	34 ± 10.2	27 ± 2.6	24 ± 2.1	23 ± 4.2	27 ± 5.1
	+	41 ± 1.8	117 ± 8.7	115 ± 8.7	86 ± 10.3	73 ± 0.9	52 ± 4.0

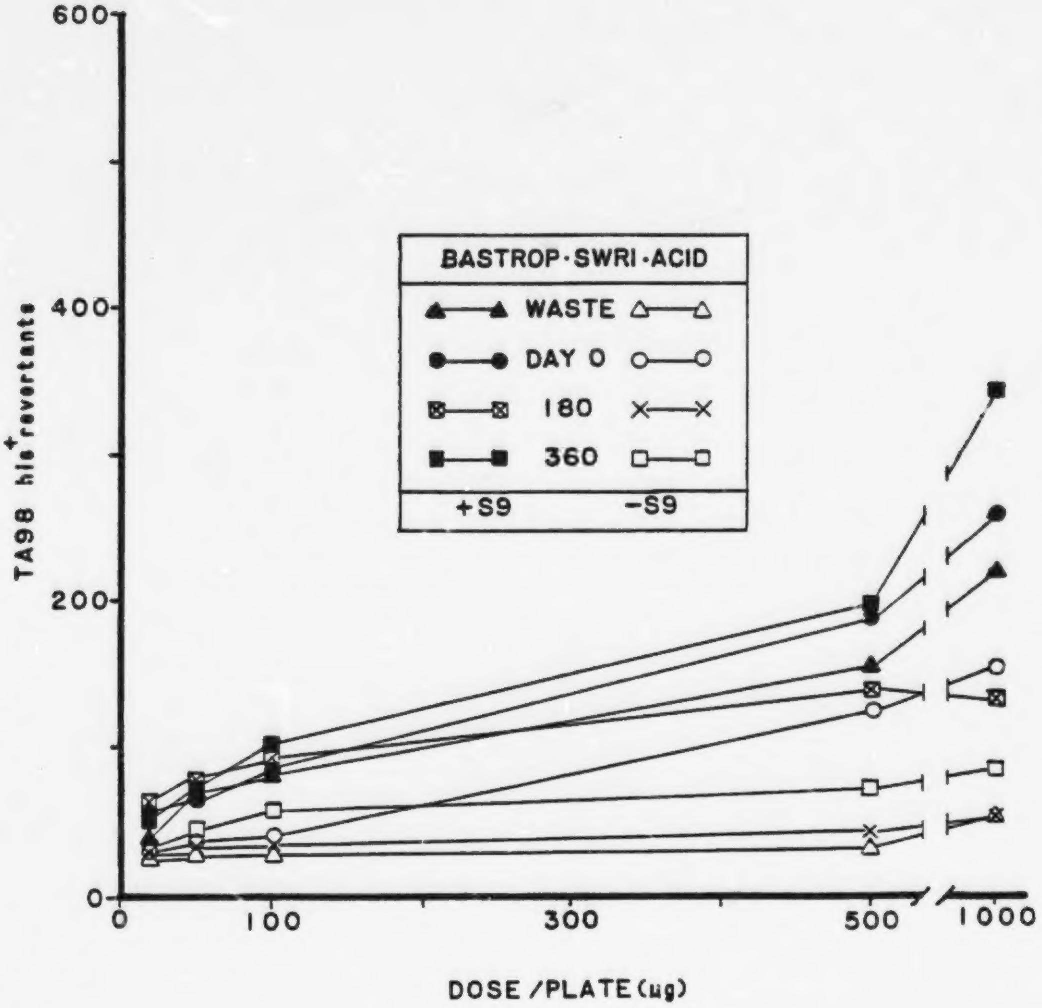


Figure 98. Mutagenic activity of acid fraction of SWRI amended Bastrop soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

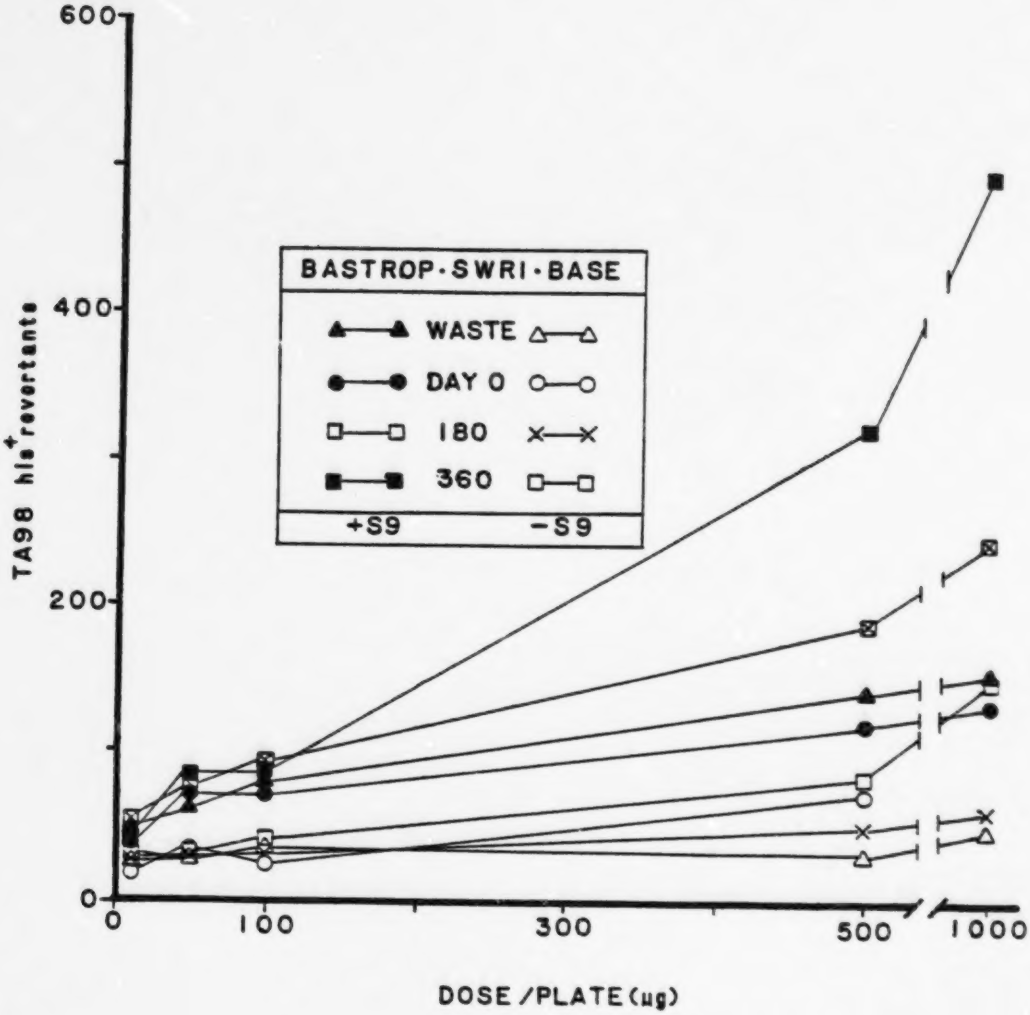


Figure 99. Mutagenic activity of base fraction of SWRI amended Bastrop soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

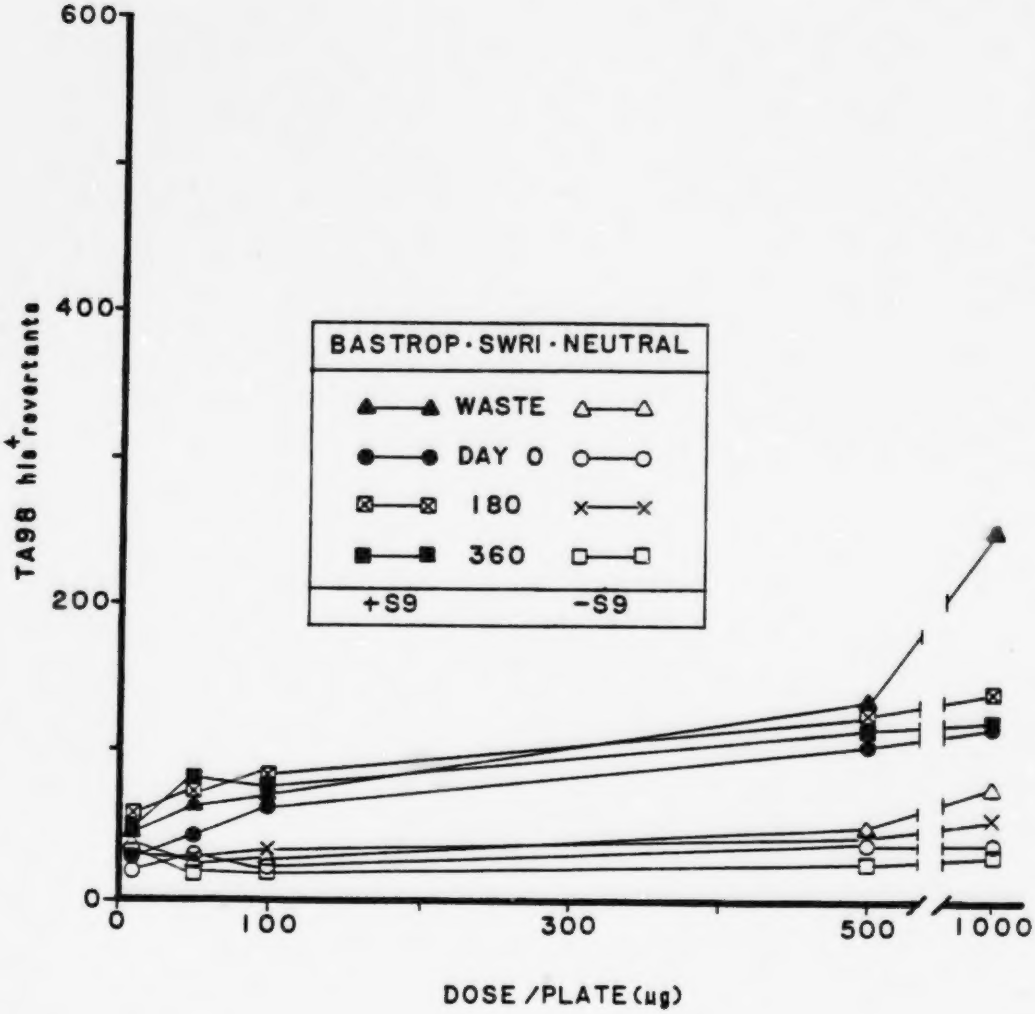


Figure 100. Mutagenic activity of neutral fraction of SWRI amended Bastrop soil measured with S. typhimurium strain TA98 with and without metabolic activation.

TABLE 85. MUTAGENIC ACTIVITY OF ACID FRACTION OF COMBO WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	33 ± 4.2	64 ± 5.6	57 ± 11.0	32 ± 6.9	30 ± 3.6	25 ± 2.0
	+	39 ± 6.8	151 ± 52.7	114 ± 15.0	83 ± 5.9	39 ± 13.7	23 ± 8.5
Day 180	-	35 ± 0.7	49 ± 11.2	42 ± 5.4	30 ± 4.7	25 ± 15.2	27 ± 1.5
	+	45 ± 2.1	133 ± 20.4	114 ± 15.4	66 ± 4.2	59 ± 3.5	56 ± 7.2
Day 360	-	24 ± 0.7	105 ± 39.3	78 ± 34.7	42 ± 18.0	35 ± 11.3	25 ± 2.2
	+	40 ± 5.8	272 ±144.5	148 ± 49.4	66 ± 17.4	51 ± 3.6	44 ± 5.6

TABLE 86. MUTAGENIC ACTIVITY OF BASE FRACTION OF COMBO WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

Day	S9	Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
		0	1	.5	.1	.05	.01
Day 0	-	33 ± 4.2	37 ± 14.2	34 ± 5.3	31 ± 10.3	26 ± 7.2	30 ± 5.1
	+	39 ± 6.8	206 ± 8.5	140 ± 33.2	87 ± 13.9	58 ± 13.1	352 ± 12.9
Day 180	-	35 ± 0.7	69 ± 17.9	55 ± 17.0	34 ± 2.2	30 ± 1.7	25 ± 1.2
	+	45 ± 2.1	205 ± 48.4	158 ± 38.0	76 ± 2.0	67 ± 5.3	55 ± 7.0
Day 360	-	24 ± 0.7	NT*	227 ± 49.0	91 ± 4.2	63 ± 4.2	31 ± 7.8
	+	37 ± 2.3	747 ± 180.0	341 ± 13.0	69 ± 18.0	59 ± 1.4	44 ± 4.9

* Not tested due to limited amount of sample available.

TABLE 87. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF COMBO WASTE AMENDED NORWOOD SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	33 ± 4.2	29 ± 6.2	26 ± 4.7	25 ± 3.2	22 ± 6.1	23 ± 4.1
	+	39 ± 6.8	97 ± 8.7	85 ± 10.8	56 ± 23.5	45 ± 9.2	39 ± 8.5
Day 180	-	35 ± 0.7	37 ± 0.9	34 ± 2.1	26 ± 2.1	28 ± 3.5	26 ± 0.3
	+	45 ± 0.9	118 ± 13.5	108 ± 1.6	69 ± 4.1	61 ± 3.9	49 ± 2.8
Day 360	-	24 ± 0.7	50 ± 28.5	40 ± 15.8	23 ± 7.5	26 ± 4.8	23 ± 1.0
	+	36 ± 2.3	148 ± 56.8	101 ± 27.0	58 ± 9.4	48 ± 6.4	40 ± 4.1

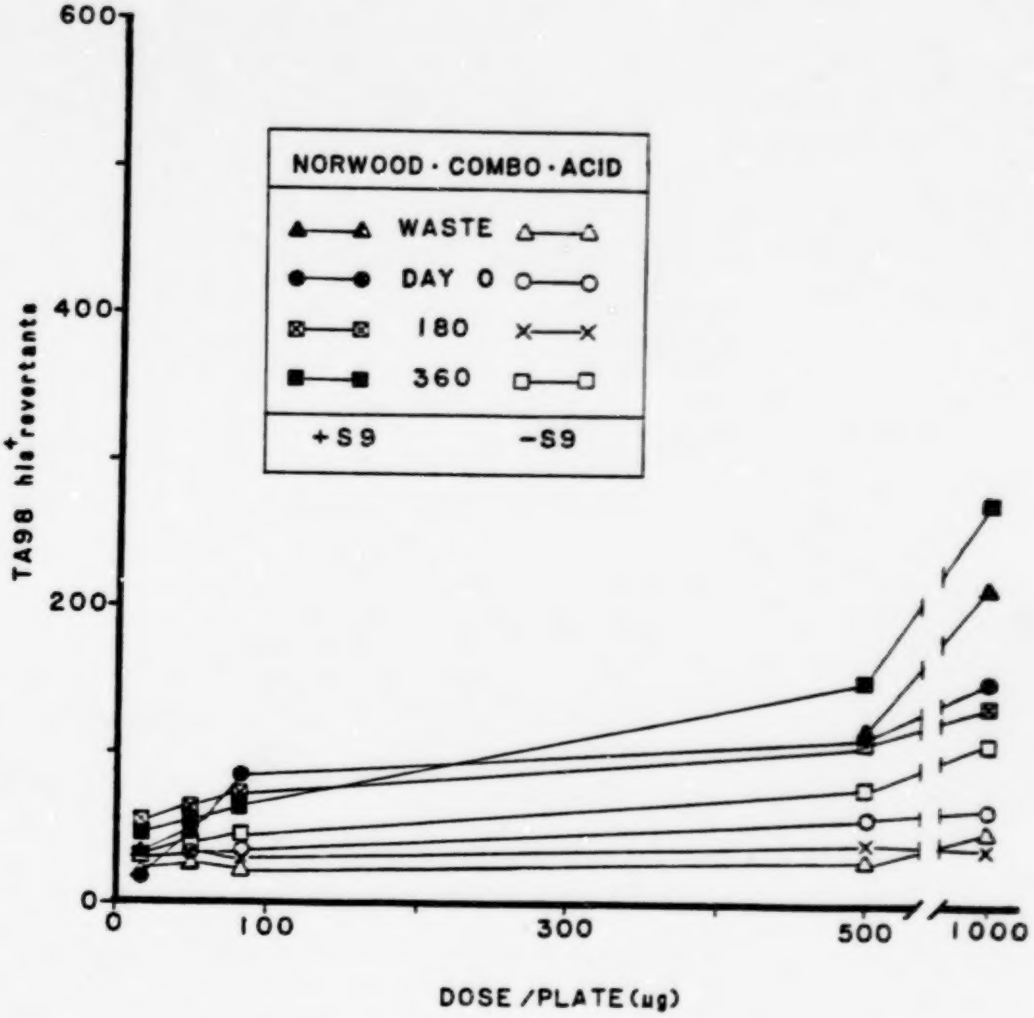


Figure 101. Mutagenic activity of acid fraction of COMBO amended Norwood soil as measured with *S. typhimurium* strain TA98 with and without metabolic activation.

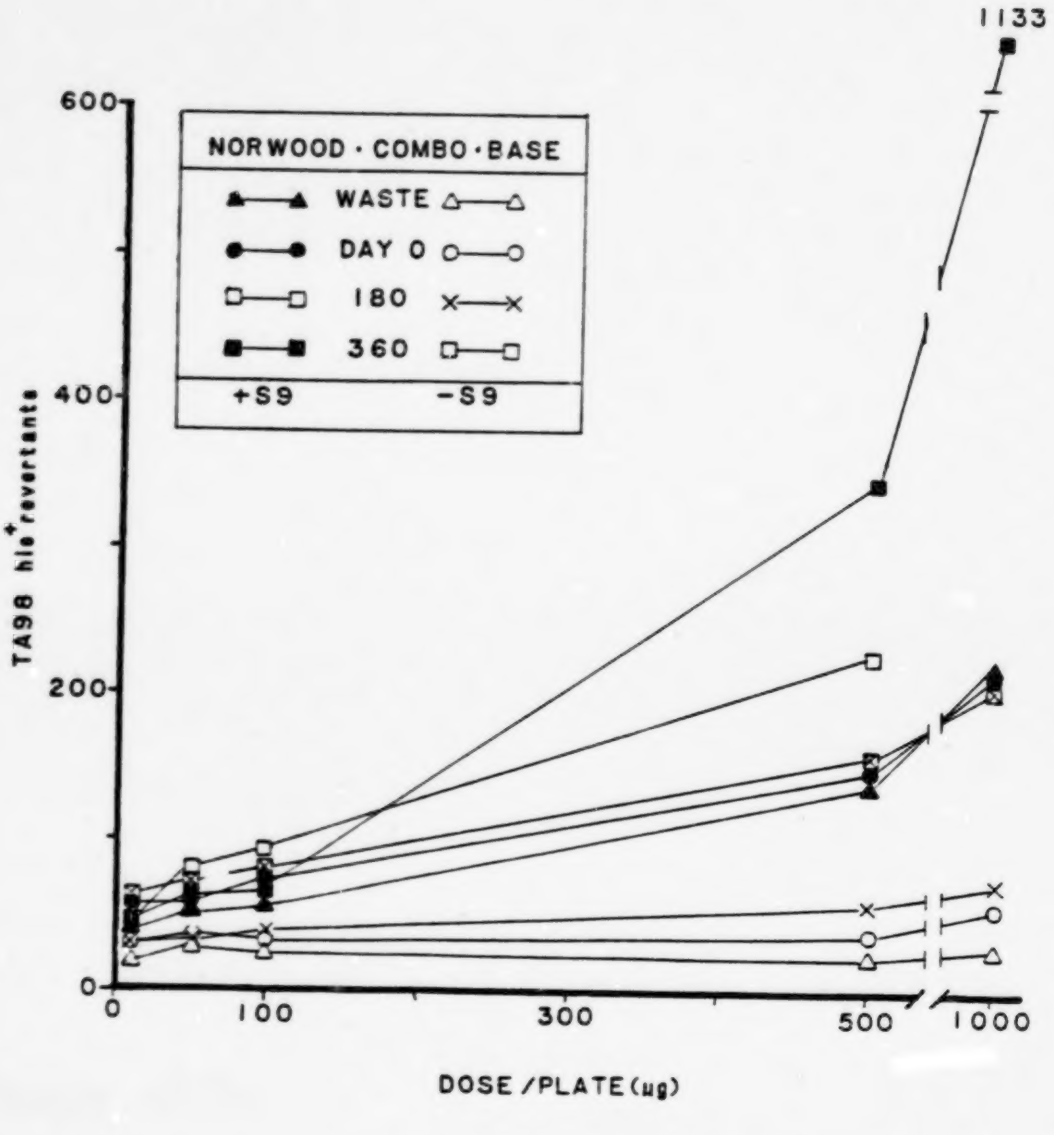


Figure 102. Mutagenic activity of base fraction of COMBO amended Norwood soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

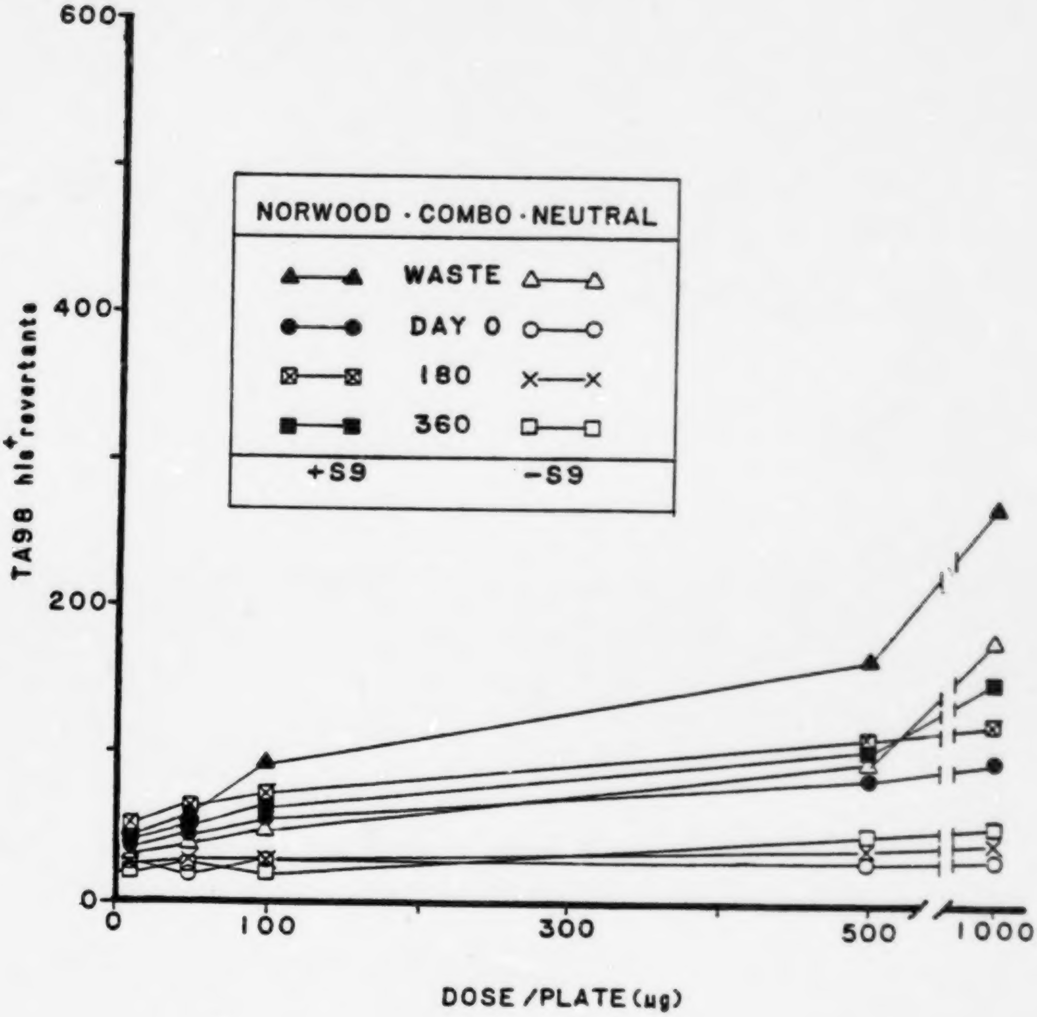


Figure 103. Mutagenic activity of neutral fraction of COMBO amended Norwood soil as measured with *S. typhimurium* strain TA98 with and without metabolic activation.

induced a response that was approximately nine times background, whereas none of the samples from earlier dates induced a response that was greater than twice background without activation. The neutral fraction of the COMBO waste amended Norwood soil induced a consistently higher response at the 1.0 mg/plate dose level from day 0 to 180 to 360. However, this increase was relatively small, and on day 360 the mutagenic potential of the extractable hydrocarbons in the neutral fractions of the COMBO waste amended Norwood soil was approximately one-half that of the neutral fraction of the waste. After 360 days of incubation in the Norwood soil, the acid and neutral fraction displayed a small but significant increase in mutagenic activity, while the base fraction displayed a dramatic increase both with and without metabolic activation.

The overall rate of hydrocarbon degradation in the COMBO waste amended Bastrop soil was much greater on day 180 and slightly greater than the rate of degradation in the Norwood soil on day 360 (Figure 82). The different rates of degradation observed in the two soils appears to have been reflected in the results from mutagenicity testing of the extracts of the waste amended Bastrop soil. In the acid fraction, the mutagenic potential of the samples collected on day 0 and day 180 were less than or equal to the mutagenic potential of the acid fraction of the waste, while the mutagenic potential of the acid fraction from the sample collected on day 360 was dramatically increased both with and without metabolic activation (Table 88 and Figure 104). In the base fraction, the mutagenic potential of the samples collected on days 180 and 360 induced at least twice the mutagenic response that was obtained from the base fraction of the waste or the day 0 sample both with and without metabolic activation (Table 89 and Figure 105). The mutagenic potential of the base fraction from day 180 without activation was equal to that of the waste sample with activation. The increase in mutagenic activity that was observed in the base fraction from day 180 may have resulted from the increased rate of degradation that occurred in the Bastrop soil. However, in the neutral fraction, there was no appreciable difference in the mutagenic potential of the waste fraction and the soil extract from day 360 (Table 90 and Figure 106). Before soil application, the neutral fraction of the COMBO waste induced a positive response in the absence of metabolic activation. In the waste amended Bastrop soil, this effect appears to have been inhibited until day 360 when direct acting mutagens were again detected (Table 90). Penalva et al. (1983) also observed that a mixture of aromatic hydrocarbons could inhibit direct acting mutagens. Therefore, as the neutral fraction accounts for 90% of the total extractable hydrocarbons in the soil, the mutagenic activity of the total residual hydrocarbons in the COMBO waste amended soils appears to be slightly increased after 360 days of soil incubation.

The alterations that were observed over time in the mutagenic potential of the three fractions of the soil-waste extracts follows strikingly similar trends. In most cases, the hydrocarbons present in

TABLE 88. MUTAGENIC ACTIVITY OF ACID FRACTION OF COMBO WASTE AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	33 ± 4.2	55 ± 21.1	41 ± 8.5	24 ± 9.0	26 ± 9.0	20 ± 6.3
	+	39 ± 6.8	94 ± 19.3	102 ± 37.8	69 ± 8.9	45 ± 6.5	30 ± 17.1
Day 180	-	32 ± 3.6	39 ± 9.0	35 ± 4.2	28 ± 4.2	25 ± 3.6	26 ± 1.2
	+	44 ± 1.4	133 ± 28.3	112 ± 14.0	64 ± 5.1	52 ± 4.9	53 ± 5.0
Day 360	-	30 ± 3.5	375 ± 30.0	283 ± 16.0	110 ± 7.8	73 ± 49.5	43 ± 7.1
	+	41 ± 7.5	1293 ± 179.0	455 ± 33.0	133 ± 141.0	65 ± 9.4	48 ± 9.2

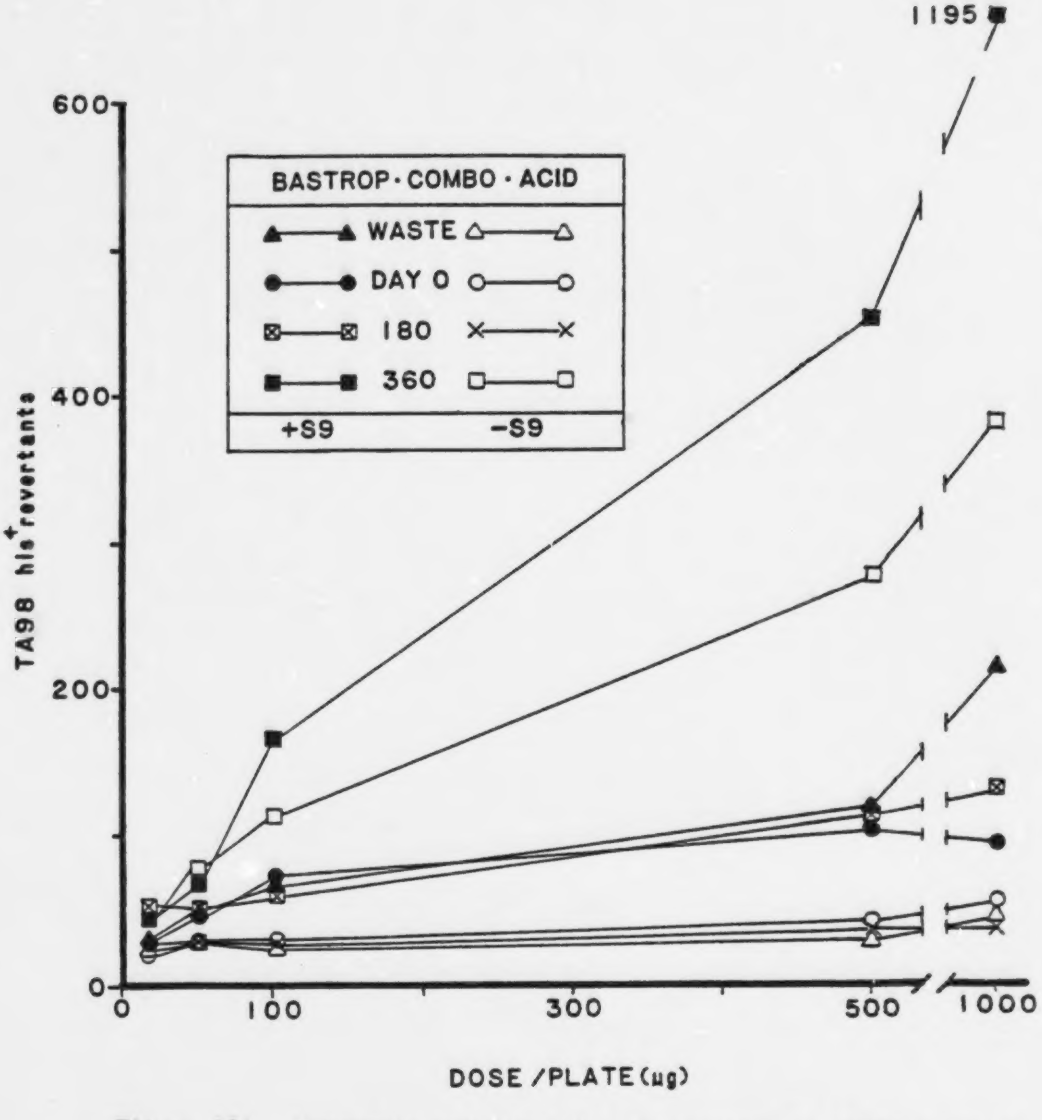


Figure 104. Mutagenic activity of acid fraction of COMBO amended Bastrop soil as measured with S. typhimurium strain TA98 with and without metabolic activation.

TABLE 89. MUTAGENIC ACTIVITY OF BASE FRACTION OF COMBO WASTE AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

		Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
Day	S9	0	1	.5	.1	.05	.01
Day 0	-	33 ± 4.2	28 ± 21.3	39 ± 37.1	27 ± 4.7	28 ± 8.7	27 ± 5.8
	+	39 ± 6.8	147 ± 13.6	149 ± 16.2	76 ± 4.9	50 ± 12.0	46 ± 7.0
Day 180	-	29 ± 6.3	221*	146*	57*	45*	26*
	+	48 ± 7.4	614*	291*	102*	70*	54*
Day 360	-	30 ± 3.5	530*	334 ± 188.0	97 ± 27.0	69 ± 12.4	61 ± 33.7
	+	41 ± 7.4	734 ± 144.9	376 ± 187.0	75 ± 47.0	58 ± 21.4	41 ± 7.9

* Mean represents average of only two samples; standard deviation not provided.

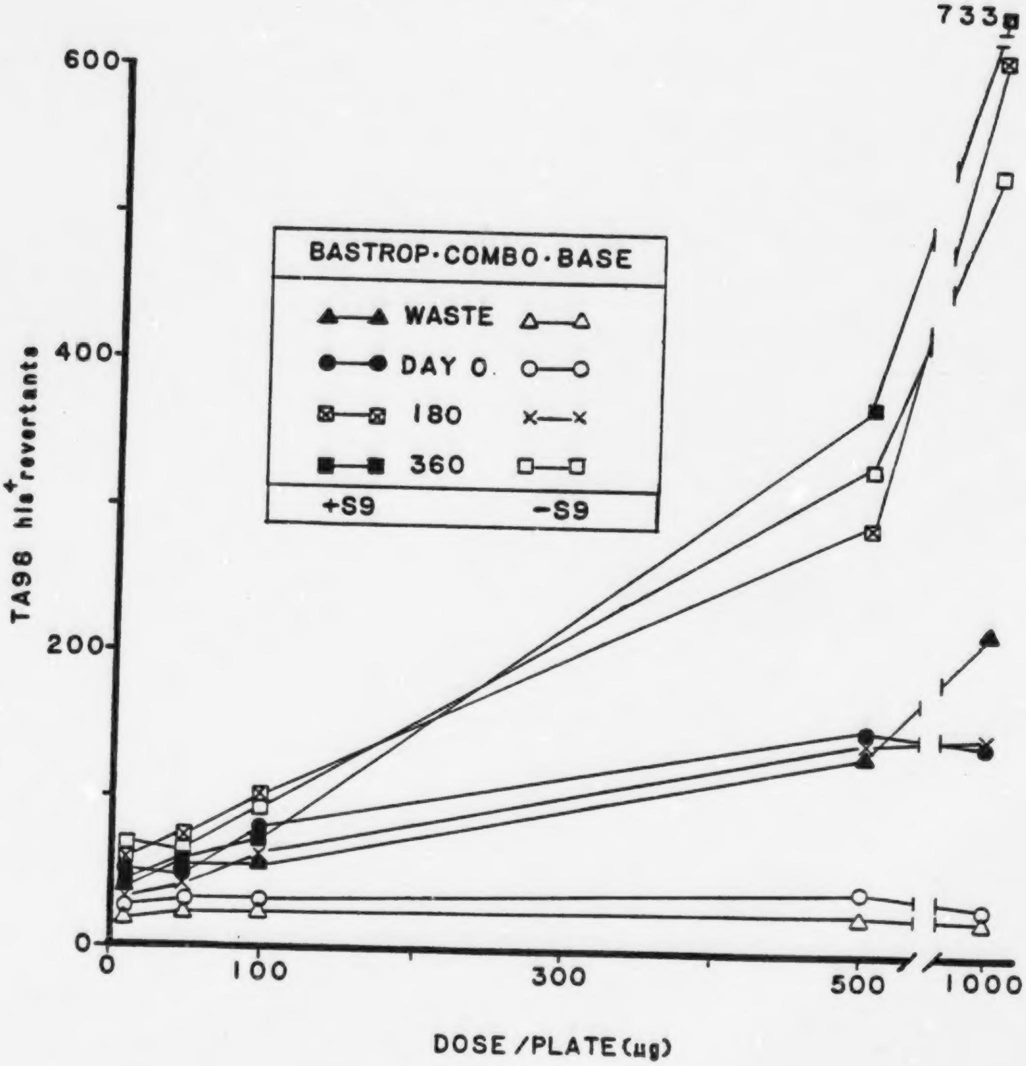


Figure 105. Mutagenic activity of base fraction of COMBO amended Bastrop soil as measured with *S. typhimurium* strain TA98 with and without metabolic activation.

TABLE 90. MUTAGENIC ACTIVITY OF NEUTRAL FRACTION OF COMBO WASTE AMENDED BASTROP SOIL AS MEASURED WITH S. TYPHIMURIUM STRAIN TA98 WITH AND WITHOUT METABOLIC ACTIVATION

Day	S9	Total TA98 his $^+$ Revertants (Mean ± S.D.)					
		Dose/Plate (mg)					
		0	1	.5	.1	.05	.01
Day 0	–	33 ± 4.2	29 ± 13.5	27 ± 10.2	23 ± 2.6	24 ± 3.9	29 ± 5.4
	+	39 ± 6.8	101 ± 11.7	105 ± 18.7	60 ± 7.9	57 ± 7.3	31 ± 10.9
Day 180	–	32 ± 3.6	35 ± 7.9	31 ± 2.9	24 ± 1.7	27 ± 3.0	28 ± 1.5
	+	44 ± 1.7	148 ± 15.0	102 ± 7.5	67 ± 9.5	64 ± 7.4	55 ± 1.5
Day 360	–	30 ± 3.5	105 ± 105.7	64 ± 40.0	37 ± 13.0	29 ± 1.6	27 ± 3.6
	+	41 ± 7.5	302 ± 279.0	145 ± 76.0	71 ± 19.0	53 ± 5.9	45 ± 11.4

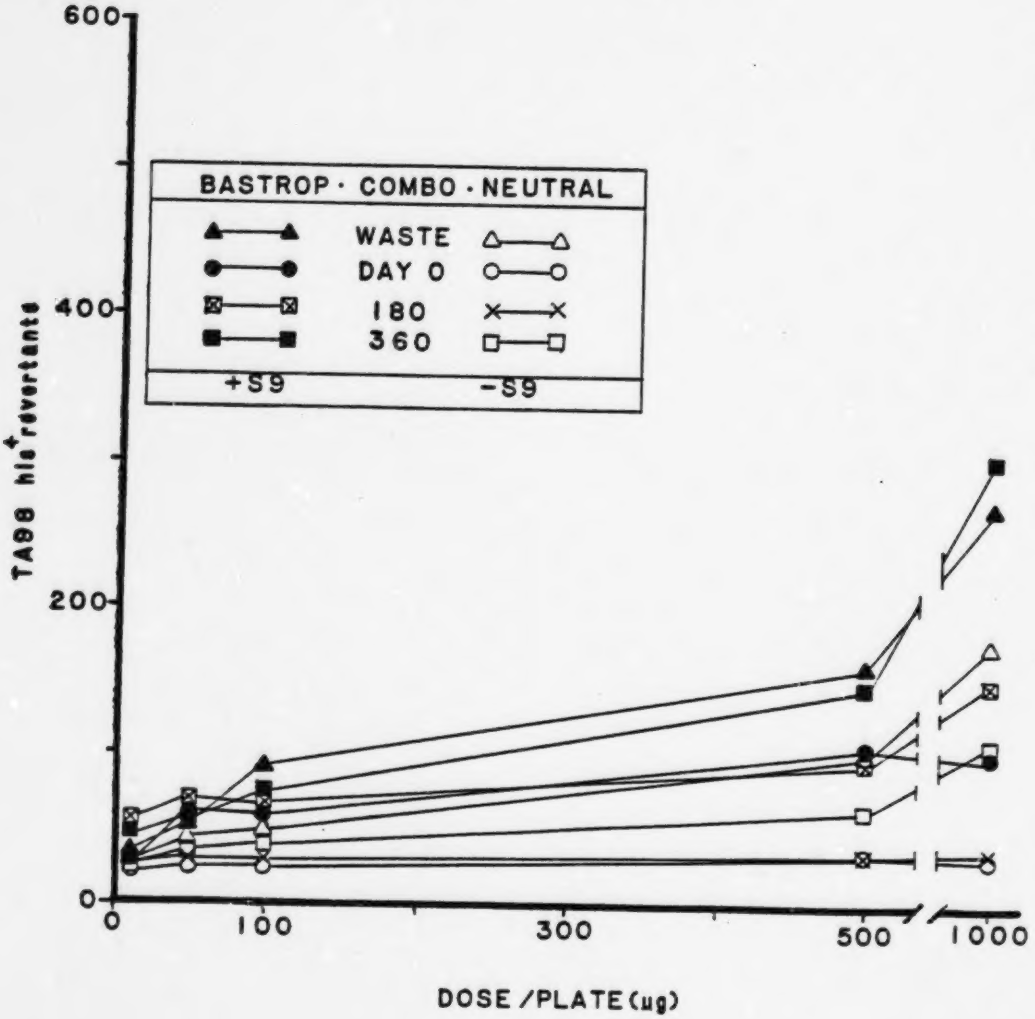


Figure 106. Mutagenic activity of neutral fraction of COMBO amended Bastrop soil as measured with *S. typhimurium* strain TA98 with and without metabolic activation.

the soil served to inhibit the mutagenic activity of the waste constituents to the extent that the activity of the soil-waste extracts from day 0 were less than the activity of the waste extracts alone. The nature of the mutagenic materials also appreared to be affected by degradation. The base fraction of all six waste-soil mixtures induced direct-acting mutagenicity at some point in time following waste application; whereas, none of the base fractions from the waste or day 0 extracts induced direct-acting mutagenicity. For the majority of the acid and base fractions, the maximum level of mutagenic activity was observed in the final sample collected. However, the mutagenic potential of the neutral fraction did not exhibit a similar increase over time and in some cases was less on day 360 than on day 0.

These results indicate that soil degradation can have a significant impact on the mutagenic potential of hazardous waste constituents. Alexander (1981) states that biodegradation may increase the toxicity of some hydrocarbons. The increases in the mutagenic activity of soil-waste extracts observed in the present study may have been a result of hydrocarbon oxidation or a result of the reduction in the number of different residual waste constituents in the soil. The initial reactions involved in the oxidation of aromatic hydrocarbons are believed to result in the formation of dioxetanes and epoxides in microbial and mammalian systems, respectively, with subsequent reactions producing dihydrodiols in both systems (Gibson, 1972). Although the epoxide and not the dihydrodiol form is mutagenic (Huberman et al., 1971), it appears from the results of the present study that biodegradation can increase the mutagenic potential of hydrocarbons in the soil. In addition, mixed function oxygenases are the enzymes believed to account for numerous mammalian activation reactions (Miller and Miller, 1974) and have also been found in plants (Higashi et al., 1981) and bacteria (McKenna, 1972; Sokatch, 1969). However, Sims and Overcash (1981) observed that the products of soil incubated benzo(a)pyrene (B[a]P) are much less mutagenic than the parent compound; and in the the present study, the degradation products of dimethyl phenanthrene appeared to be less mutagenic than the original components of the neutral fraction of the COMBO waste. Thus, the major impact of degradation on the mutagenicity of a complex mixture may be its affect on the composition of the mixture.

The components of a complex mixture may have synergistic, antagonistic, or additive effects on the mutagenic potential of the mixture as a whole. Hass et al. (1981) suggest that even a very slight shift in the concentration of one of the nonmutagenic chemical species can drastically alter the response of the mutagenic species. Hermann (1981) observed that several nonmutagenic hydrocarbons enhanced the mutagenic activity of B(a)P, while most mutagenic polycyclic aromatic hydrocarbons (PAH) produced a large decrease in the mutagenicity of benzo(a)pyrene. When Penalva (1983) examined the interactions of the vapors and aerosols emitted by road coating tar, they found enhancement at low levels and inhibition at high levels for indirect-acting mutagens. However, only the inhibition effect appeared for direct acting mutagens (Penalva, 1983). This may explain the inhibition of direct-acting mutagenicity that was observed in the initial extracts from the COMBO waste amended soil.

The data of Shahin and Fournier (1978) indicate that the PAH fraction of Athabasca tar-sand suppressed the mutagenicity of 2-aminoanthracene. In a study by Kaden et al. (1979) the mutagenic activity of a kerosene soot extract was 10 to 20 times higher than could be accounted for by the amount of B(a)P present. By evaluating the mutagenic activity of the 70 PAH identified in the soot, Kaden et al. (1979) were able to account for the mutagencity of the whole PAH fraction in terms of the additive mutagenic activity of the individual components. Haugen and Peak (1983) observed that a complex mixture of aromatic hydrocarbons isolated from a coal-derived oil suppressed the mutagenic activity of indirect mutagens but had no effect on the activity of direct-acting mutagens. Haugen and Peak (1983) also demonstrated that this suppression was produced by inhibition of the microsomal monooxygenase system. Environmental factors may have also affected the mutagenic activity of the residual hydrocarbons (McCoy et al., 1979; Claxton and Barnes, 1982). The information in the literature indicates that the increased mutagenicity observed in some of the soil-waste extracts from the present study was probably a result of degradation reducing the number of compounds in the complex mixtures. This reduction effectively reduced competition for activating enzymes with a resultant increase in the mutagenicity of the residual compounds. While this reduction in compounds appears to be the dominant factor affecting the mutagenic activity of hazardous waste amended soils, the increased presence of direct-acting mutagens with time indicates microbial oxidations may also have played an important role.

The results of the present study and those in the literature indicate that the composition of a complex mixture will have a significant influence on the mutagenic potential of the mixture. The composition of the acid, base, and neutral fractions from the PENT S waste amended Norwood and Bastrop soils are given in Table 91 and Figures 107 through 117. Apparently from the variety of constituents identified in the extracts, the prediction of the genotoxic effects of these samples from chemical analysis alone would be difficult if not impossible. However, from the variety of identified initiators, promotors, mutagens, and carcinogens, chemical analysis evidently provides valuable information pertinent to the interpretation of the results from the biological testing. A number of alkanes were identified in the base and neutral fractions of the soil extracts. These included the promoting agents dodecane, tetradecane, and octadecane; the cocarcinogens octadecane and eicosane; and hexadecane which is an inhibitor (Lankas et al., 1978; Goldschmidt, 1981). The majority of the alkanes detected in the PENT S soil extracts were also present in the extract of the unamended soil.

TABLE 91. LIST OF COMPOUNDS DETECTED IN PENT S WASTE AMENDED SOIL

Sample	Fraction		
	Acid Compound (Peak Number)*	Base Compound (Peak Number)*	Neutral Compound (Peak Number)*
Norwood Day 0	Not Determined	Methyl napthalene (249)*M1; CO Dimethyl napthalene (289)*MO Dihydro acenaphthylene (314)*M1 Hexene (332) Dimethyl butane (358) Unknown (377) Epoxy-methyl-pentane (397) Unknown (435) Unknown (461) Trifluoro methane (472)*M1 Unknown (496) Unknown (523) Unknown (541) Unknown (570) Unknown (590) Unknown (616) Unknown (646) Unknown (676)	Unknown (238) Methyl napthalene (251) (257)*M1; CO Biphenyl (281)*MO; Cl Dimethyl napthalene (291) (295)*MO Dihydro acenaphthylene (321)*M1 Dibenzofuran (334) Fluorene (359)*MO; CO Unknown (384) Unknown (406) Dibenzothiophene (424)*MO Phenanthrene (432)*MO; CO Unknown (462) Unknown (478) Unknown (503) Fluoranthene (531)*M1; CO; CC Pyrene (547)*M1; CO; CC Unknown (589) Unknown (615) Unknown (637)
Norwood Day 360	Unknown (267) Dimethyl nonane (303) Unknown (377) Methyl propyl pentanol (358) Unknown (377) Ethyl hexanol (399) Pentachlorophenol (419)*MO; Cl Dimethyl undecane (437) Trimethyl octane (469) Benzene dicarboxylic acid (492)*MO Fluoranthene (522)*M1; CO; CC Pyrene (538)*M1; CO; CC Unknown (562) Unknown (591) Unknown (620) Unknown (639) Unknown (657)	Unknown (277) Unknown (303) Ethyl heptane (318) Unknown (339) Dimethyl nonane (359) Unknown (378) Dimethyl hexane (399) Trimethyl heptane (469) Propyl aziridine (492) Fluoranthene (523)*M1; CO; CC Pyrene (539)*M1; CO; CC Unknown (586) Unknown (619) Unknown (650) Unknown (681)	Unknown (237) Unknown (271) Unknown (281) Alkane (308) Dihydro acenaphthylene (324)*M1 Unknown (334) Trimethyl naphthalene (347)*MO Unknown (375) Dimethyl octane (384) Dimethyl biphenyl (407) Unknown (421) Methyl propyl pentanol (445) Unknown (462) Trimethyl octane (476) Cyclopenta phenanthrene (481)*MO Unknown (499) Dimethyl undecane (508) Fluoranthene (529)*M1; CO; CC Pyrene (544)*M1; CO; CC Unknown (568)

TABLE 91 CONTINUED

Sample	Fraction		
	Acid Compound (Peak Number)*	Base Compound (Peak Number)*	Neutral Compound (Peak Number)*
Bastrop Day 0	Methyl napthalene (239)*M1; CO	Methyl napthalene (240)*M1; CO	Unknown (220)
	Unknown (246)	Dimethyl napthalene (285) (299)*MO	Methyl naphthalene (244) (250)*M1; CO
	Dimethyl napthalene (282) (292)*MO	Dihydro acenaphthylene (317)*M1	Unknown (277)
	Dihydro acenaphthylene (319)*M1	Methyl ethyl napthalene (326)	Dimethyl naphthalene (287) (293)*MO
	Alkane (326) (367) (407)	Dibenzofuran (331)	Dihydro acenaphthylene (319)*M1
	Dibenzofuran (332)	Unknown (343)	Alkane (323)
	Trimethyl napthalene (346)*MO	Phenalene (352)*M1	Dibenzofuran (332)
	Phenalene (353)*M1	Trimethyl napthalene (363)*MO	Trimethyl naphthalene (337) (343)*MO
	Pentachlorophenol (429)*MO; Cl	Unknown (365)	Phenalene (358)
	Unknown (481)	Alkane (405)	Alkane (364)
	Unknown (515)	Dibenzothiophene (424)*MO	Unknown (378)
	Fluoranthene (535)*M1; CO: CC	Methyl dibenzothiophene (461)*M1	Unknown (392)
	Pyrene (553)*M1; CO: CC	Methyl phenanthrene (478)*M1	Methyl fluorene (405)
	Unknown (635)	Phenyl napthalene (504)	Dibenzothiophene (422)*MO
	Unknown (691)	Fluoranthene (533)	Unknown (438)
	Unknown (728)	Pyrene (550)	Methyl dibenzothiophene (467)*MO
	Unknown (770)	Unknown (627)	Methyl phenanthrene (477)*MO
	Unknown (807)	Unknown (679)	Cyclopenta phenanthrene (482)*MO
	Unknown (846)	Unknown (730)	Phenyl napthalene (501)
	Unknown (884)	Unknown (775)	Unknown (511)
		Unknown (822)	Fluoranthene (531)*M1; CO
		Unknown (876)	Pyrene (548)*M1; CO
			Unknown (561)
			Unknown 578)
Bastrop Day 360	Unknown (267)	Dimethyl hexane (277)	Unknown (276)
	Ethyl methyl pentanol (277)	Unknown (303)	Tetradecane (287)*P
	Unknown (303)	Dihydro acenaphthylene (321)*M1	Unknown (314)
	Dihydro acenaphthylene (323)*M1	Unknown (343)	Dihydro acenaphthylene (335)*M1
	Trimethyl napthalene (345)*MO	Unknown (361)	Trimethyl naphthalene (359)*MO
	Dodecane (358)*P; CC	Trimethyl octane (397)	Unknown (385)
	Dimethyl octane (376)*P	Unknown (456)	Hexadecane (400)*A
	Dimethyl undecane (398)	Cyclopenta phenanthrene (476)*MO	Trimethyl octane (417) (447)
	Pentachlorophenol (422)*Cl	Unknown (502)	Heptadecane (429)

TABLE 91 CONTINUED

Sample	Fraction		
	Acid Compound (Peak Number)*	Base Compound (Peak Number)*	Neutral Compound (Peak Number)*
	Methyl propyl nonane (432) (467) (498)	Dimethyl phenanthrene (514)*MI	
	Phenyl naphthalene (452)	Fluoranthene (521)*MI; CO; CC	Octadecane (450)*P; CC
	Cyclopenta phenanthrene (473)*MO	Pyrene (537)*MI; CO; CC	Unknown (467)
			Nonadecane (479)
	Unknown (510)		Cyclopentaphenanthrene (487)*MO
	Fluoranthene (519)*MI; CO; CC		Eicosane (510) *CC
	Pyrene (534)*MI; CO; CC		Fluoranthene (531)*MI; CO; CC
	Unknown (558)		Heneicosane (541)
			Pyrene (547)*MI; CO; CC
			Docosane (571)
			Unknown (601) (630)

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* Potential genetic toxicity: I = initiator; P = promotor; A = antagonist; CC = cocarcinogen(synergist); MO = nonmutagenic; MI = mutagenic; CO = non-carcinogen; CI = carcinogen; all others = no information. The references used in determining these factors include McCann _et al_ (1975); Kaden _et al_ (1979); Goldschmidt (1981) and DHEW (1969).

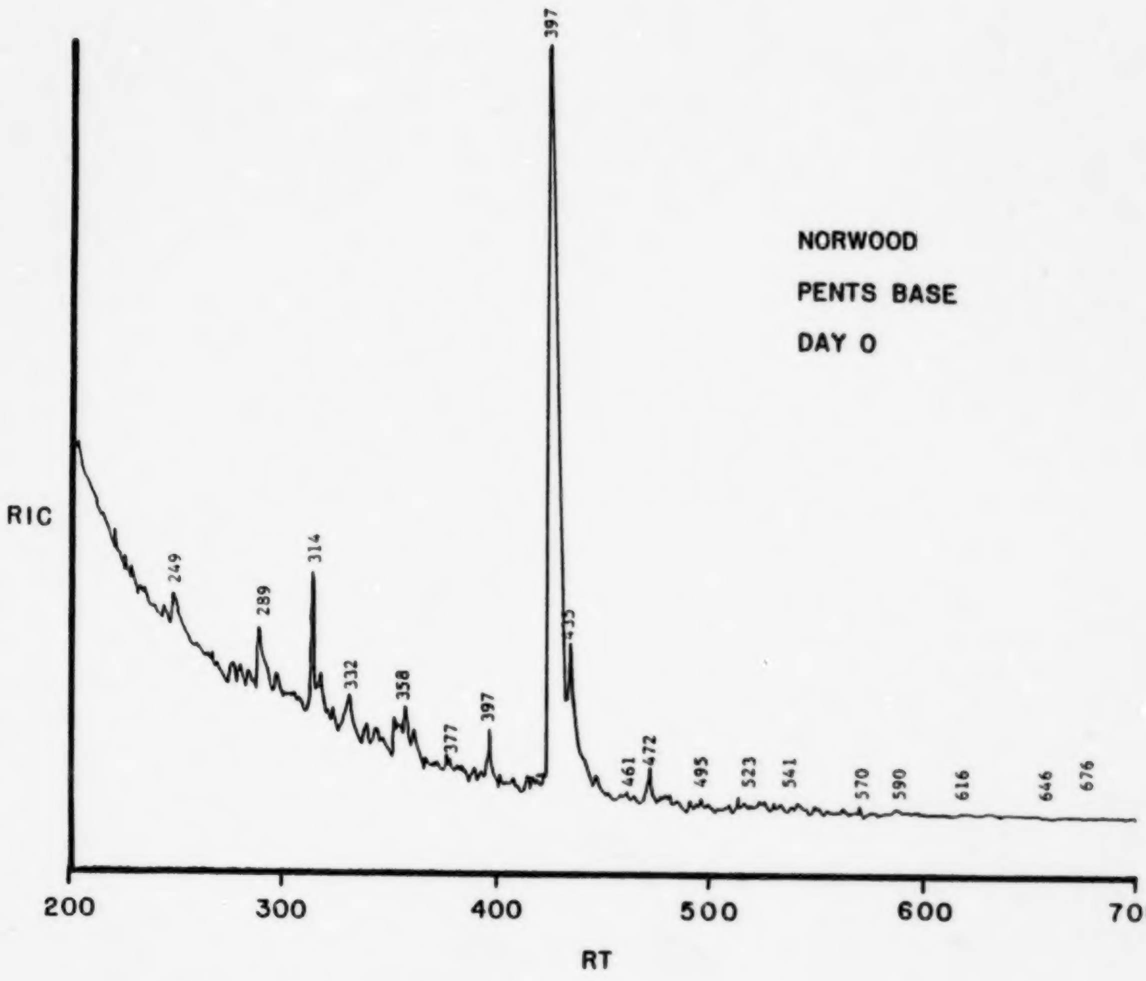

Figure 107. GC/MS chromatograph of base fraction of PENT S amended Norwood soil collected on day 0.

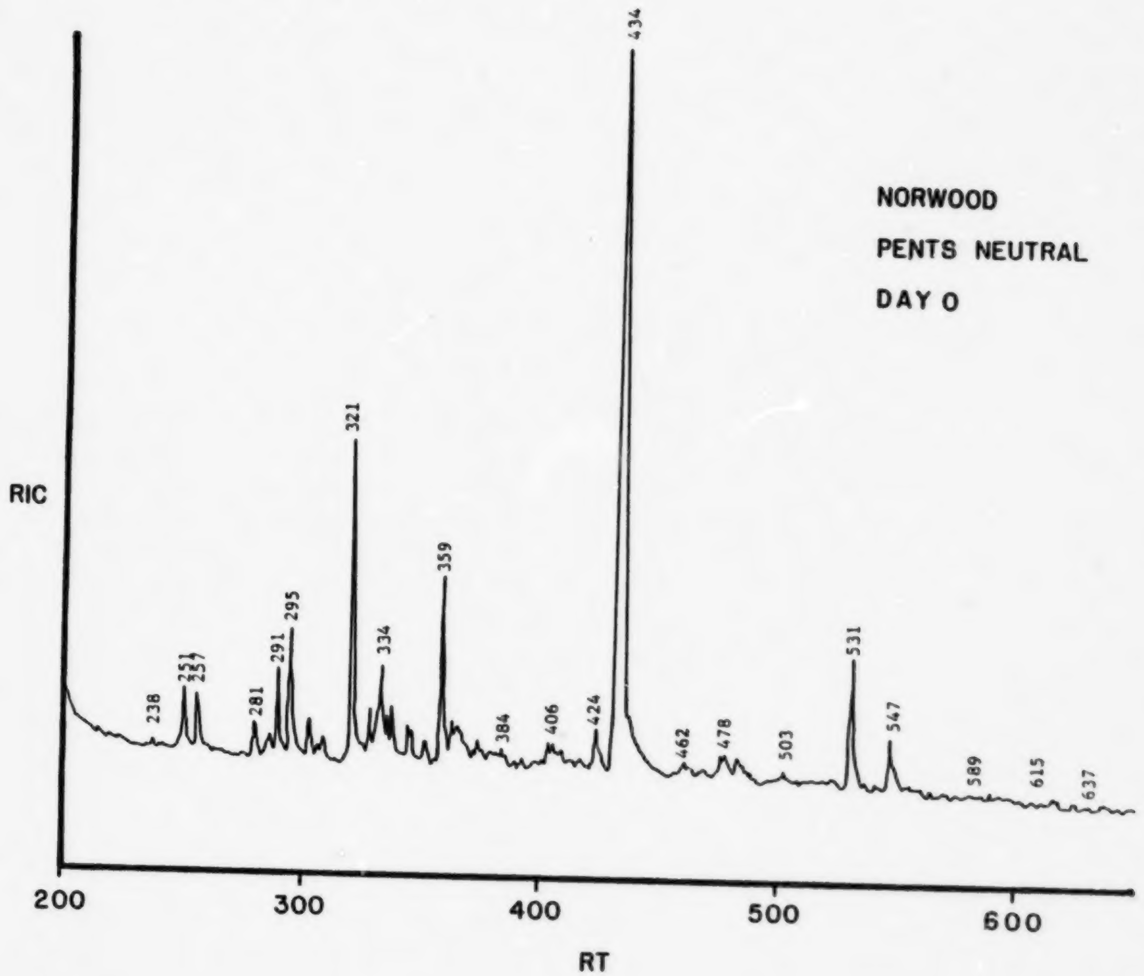

Figure 108. GC/MS chromatograph of neutral fraction of PENT S amended Norwood soil collected on day 0.

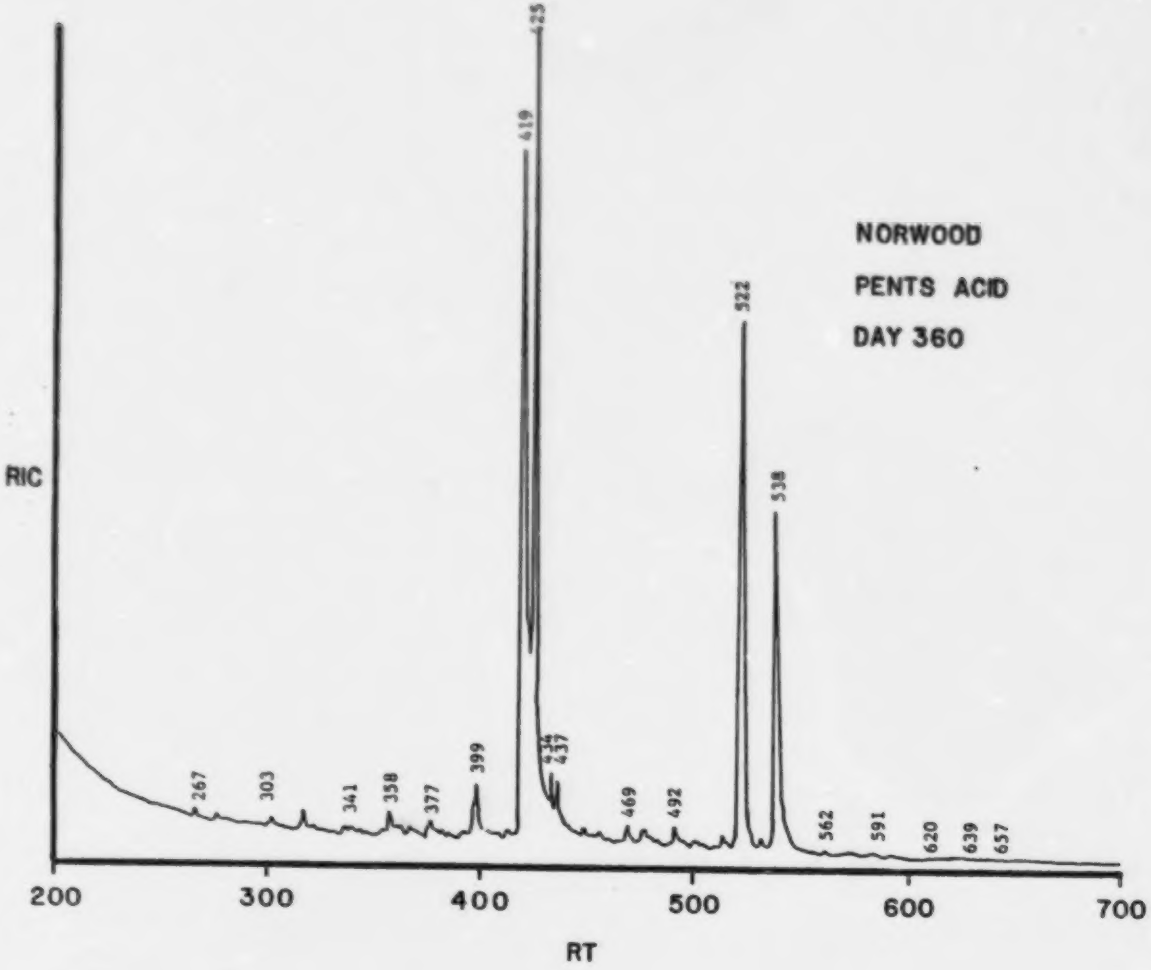

Figure 109. GC/MS chromatograph of acid fraction of PENT S amended Norwood soil collected on day 360.

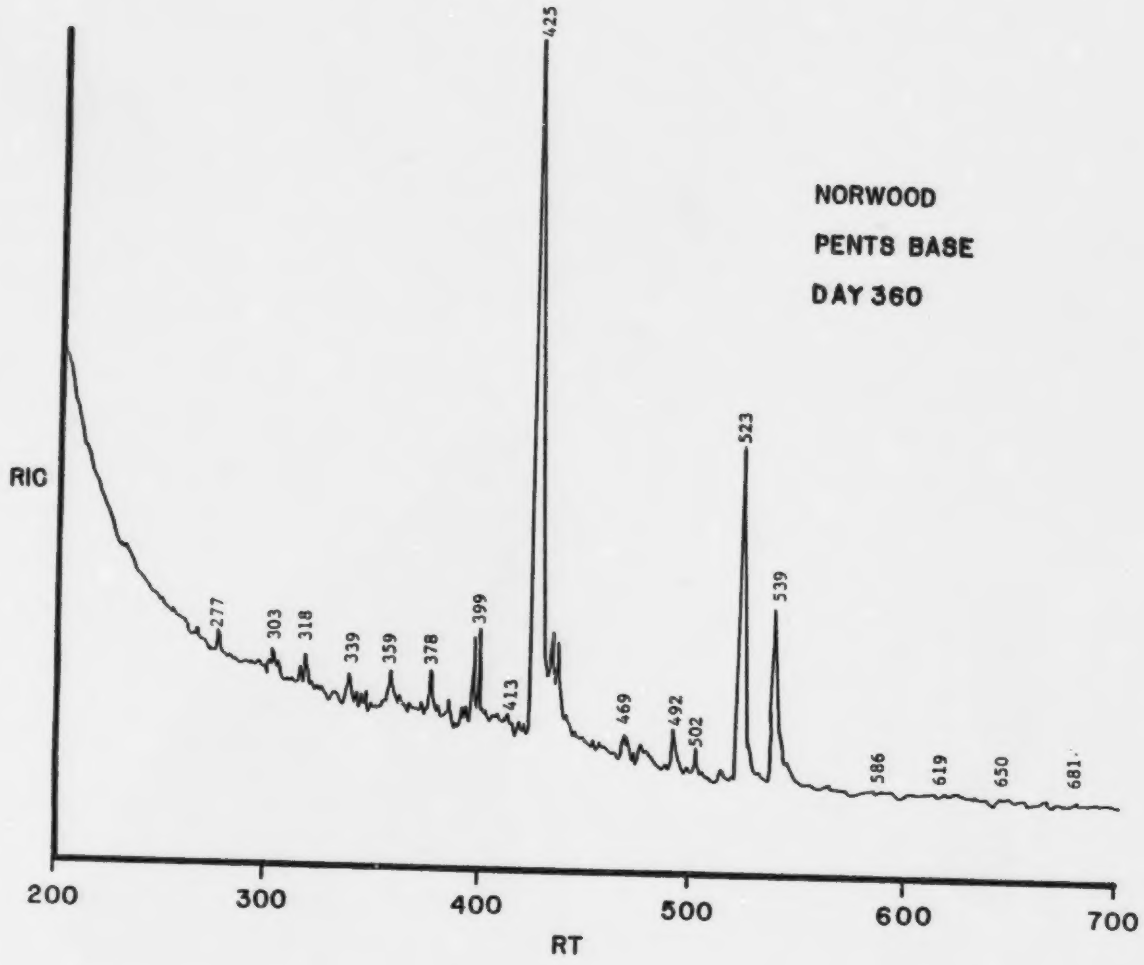

Figure 110. GC/MS chromatograph of base fraction of PENT S amended Norwood soil collected on day 360.

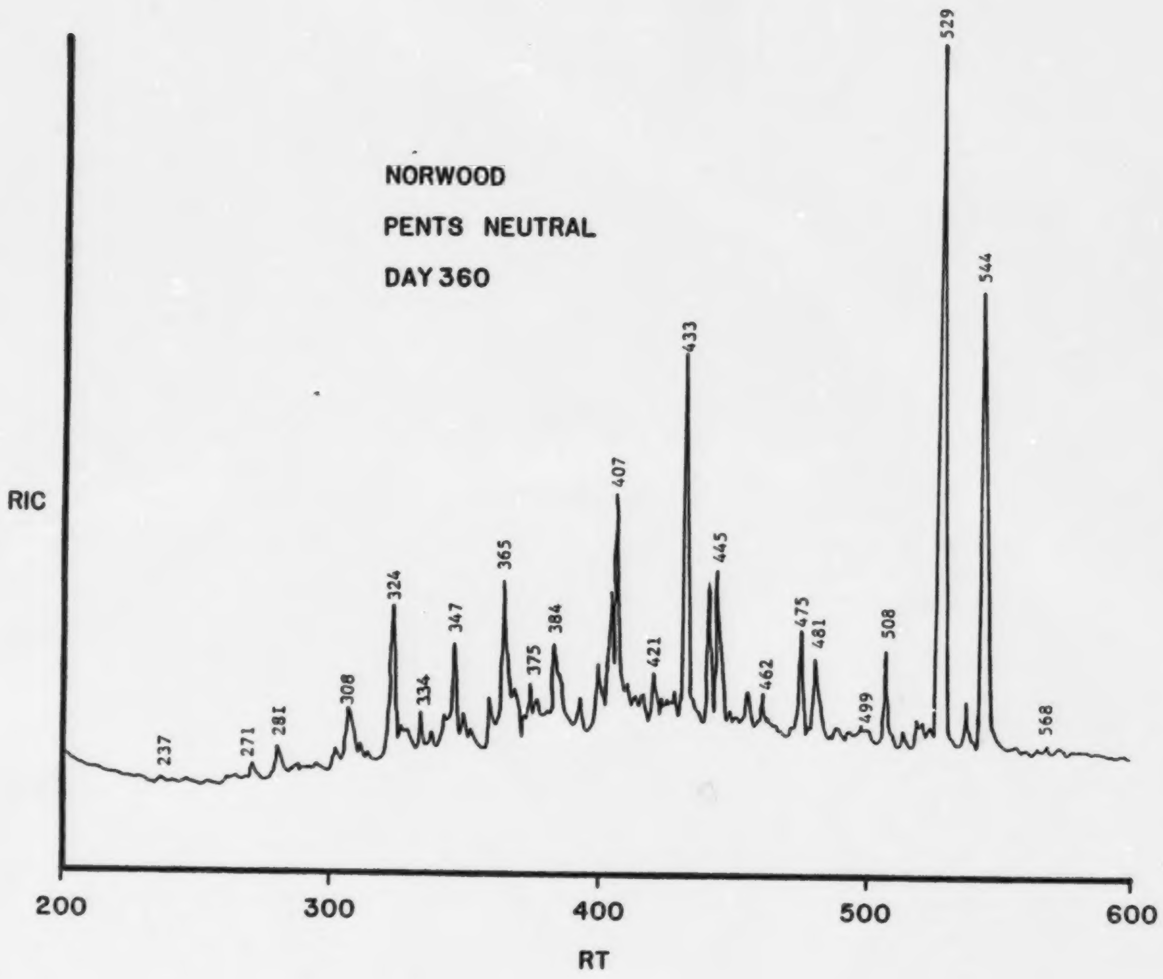

Figure 111. GC/MS chromatograph of neutral fraction of PENT S amended Norwood soil collected on day 360.

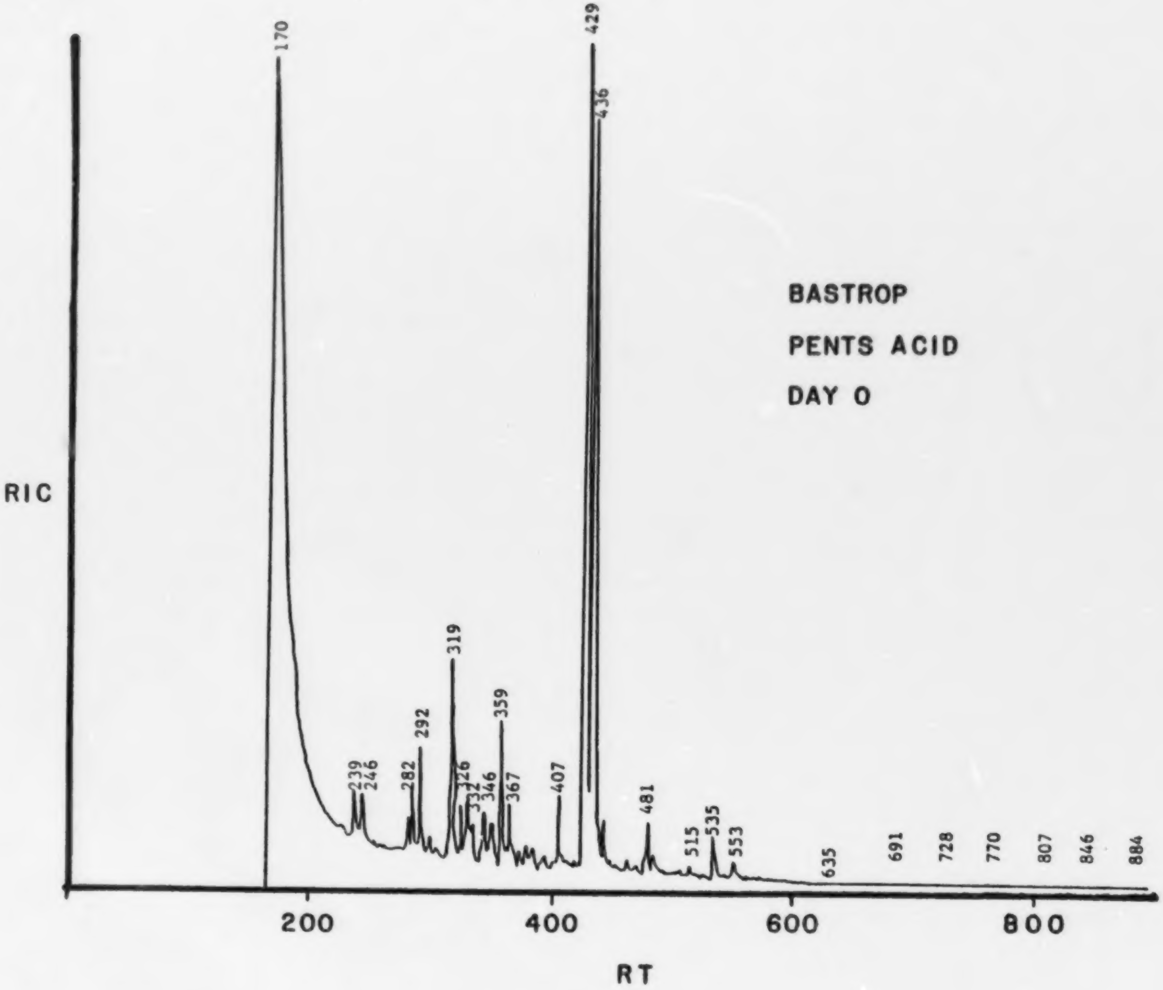

Figure 112. GC/MS chromatograph of acid fraction of PENT S amended Bastrop soil collected on day 0.

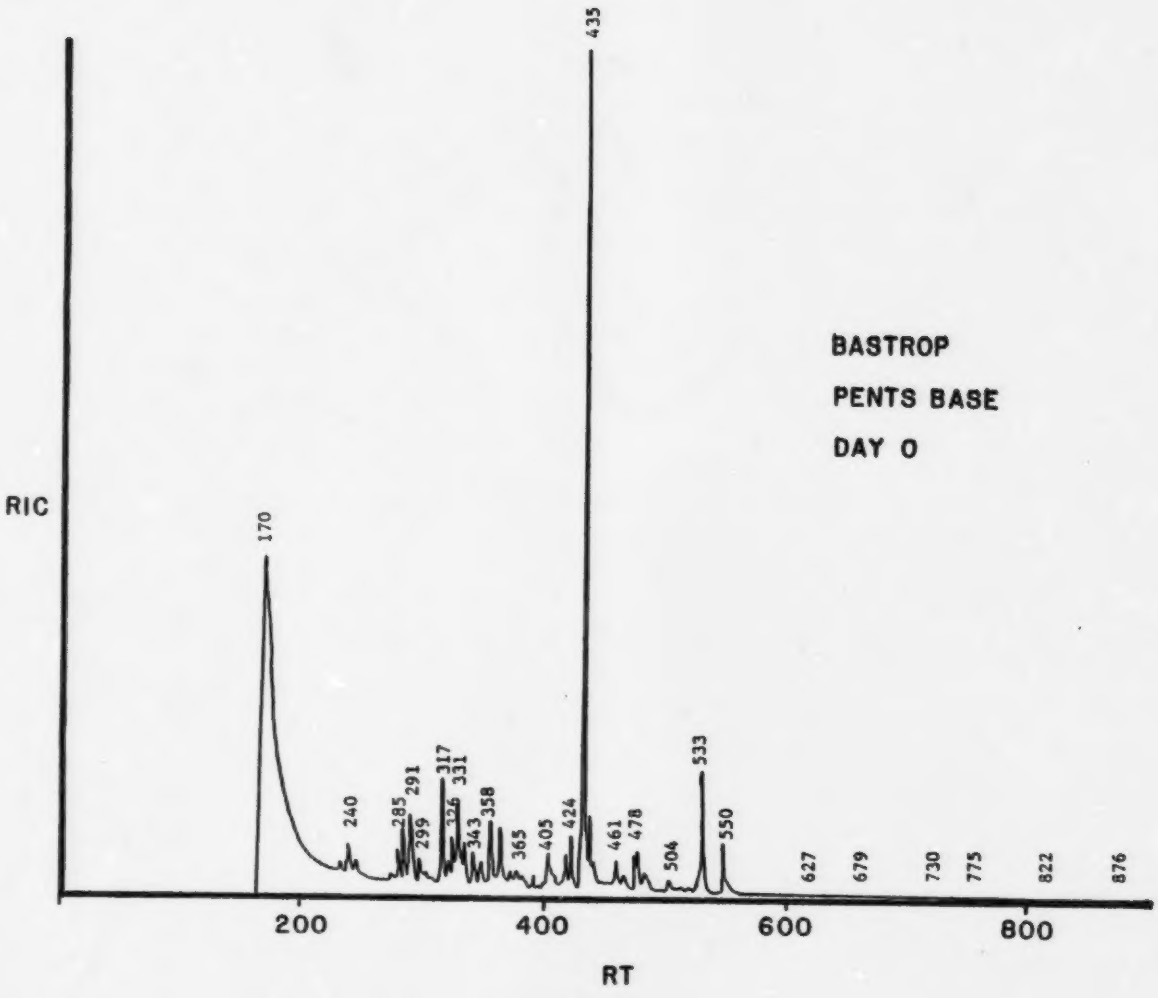

Figure 113. GC/MS chromatograph of base fraction of PENT S amended Bastrop soil collected on day 0.

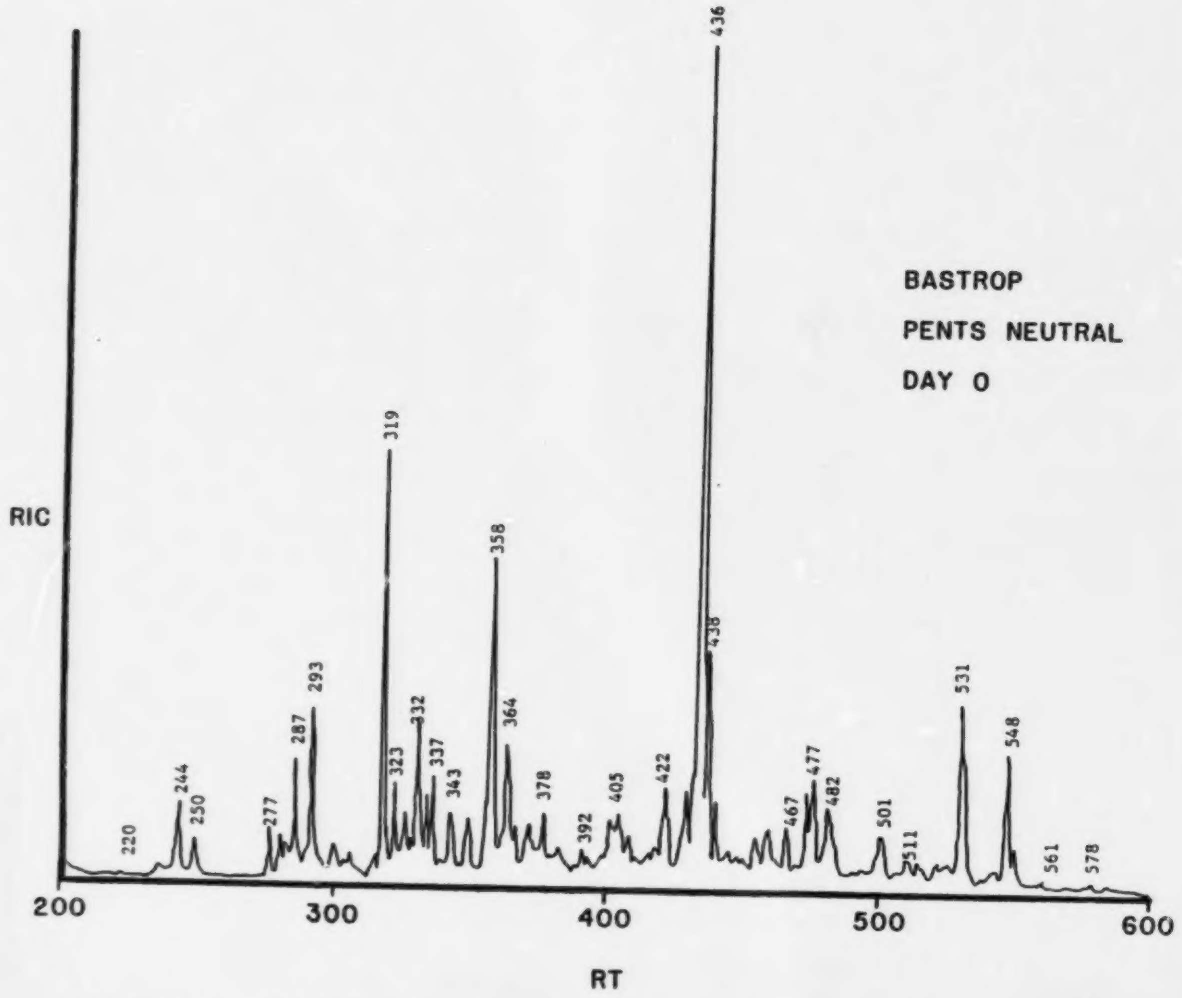

Figure 114. GC/MS chromatograph of neutral fraction of PENT S amended Bastrop soil collected on day 0.

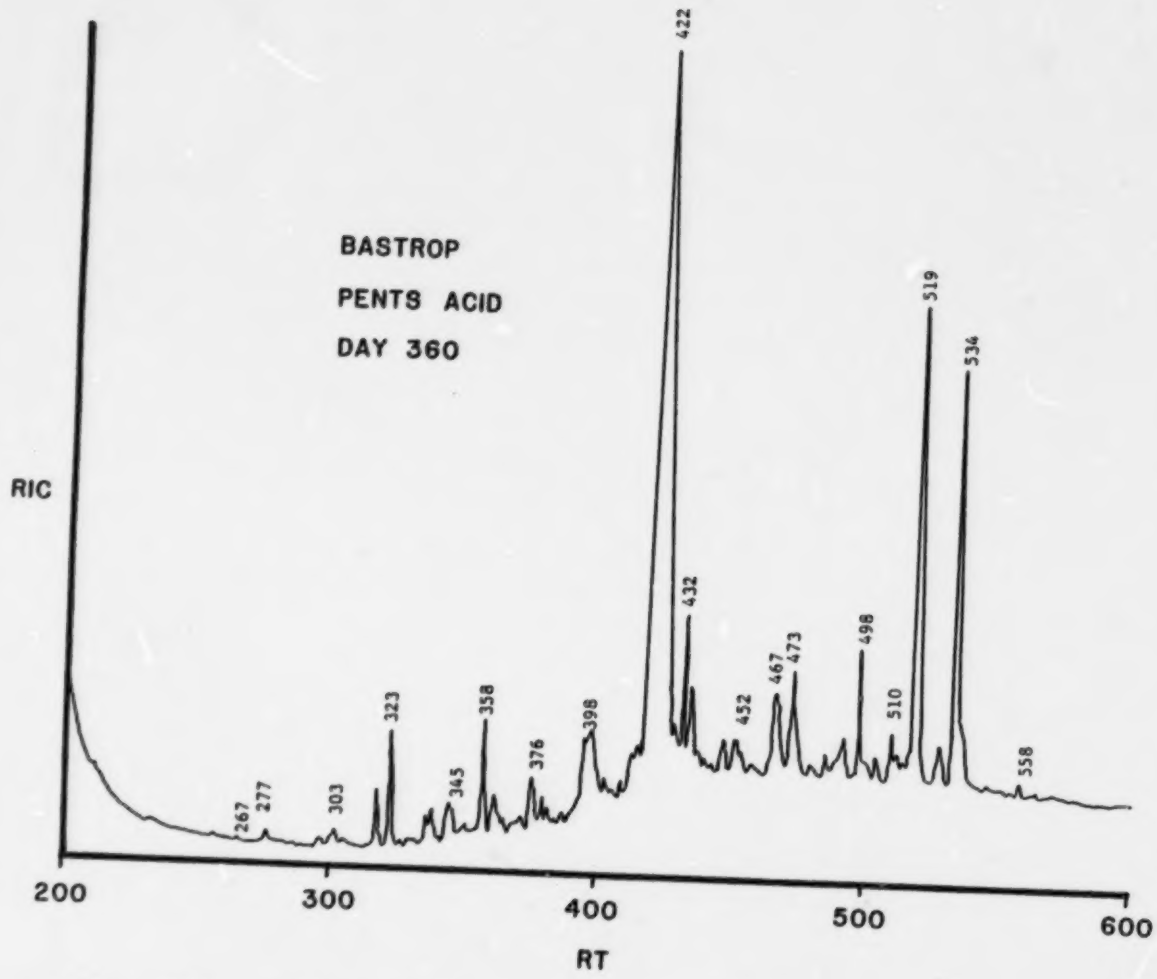

Figure 115. GC/MS chromatograph of acid fraction of PENT S amended Bastrop soil collected on day 360.

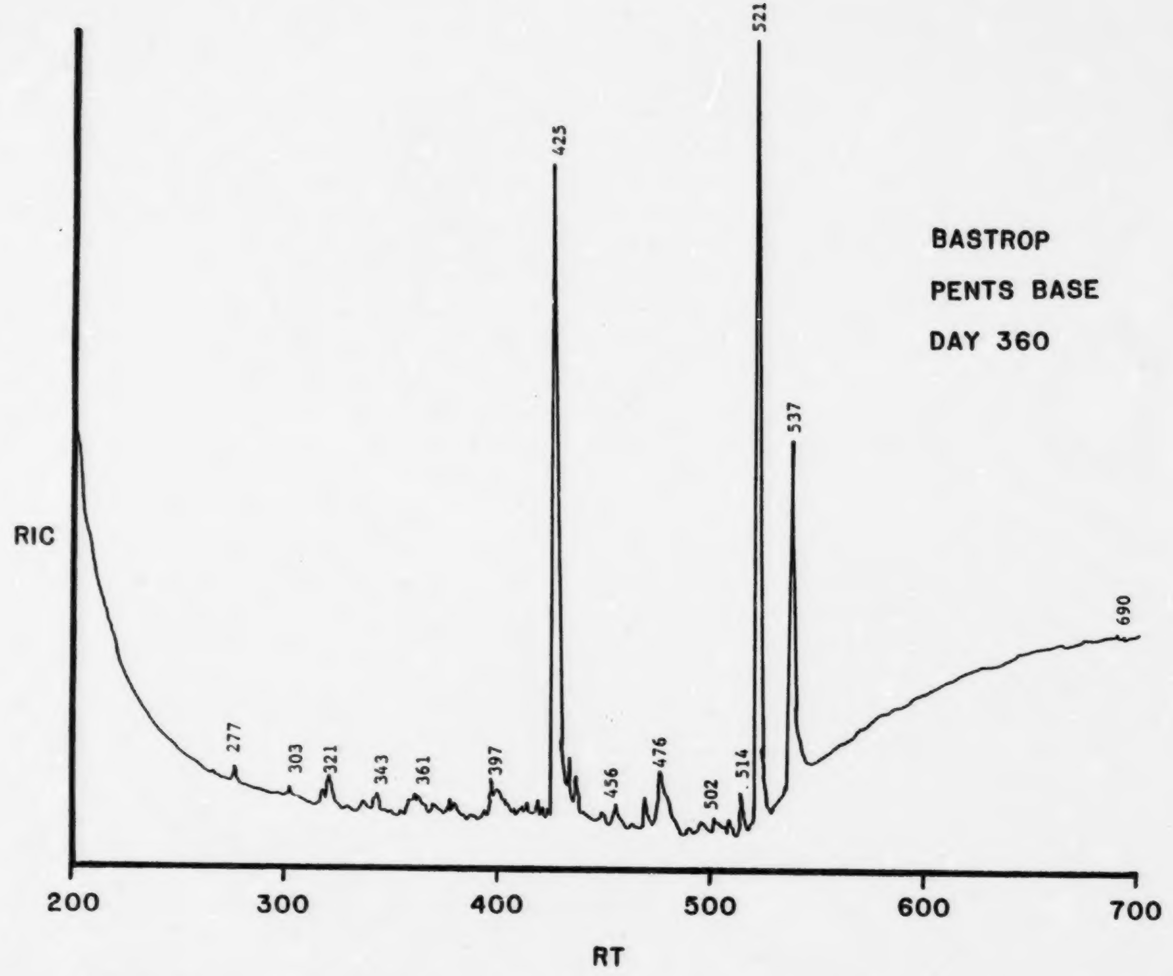

Figure 116. GC/MS chromatograph of base fraction of PENT S amended Bastrop soil collected on day 360.

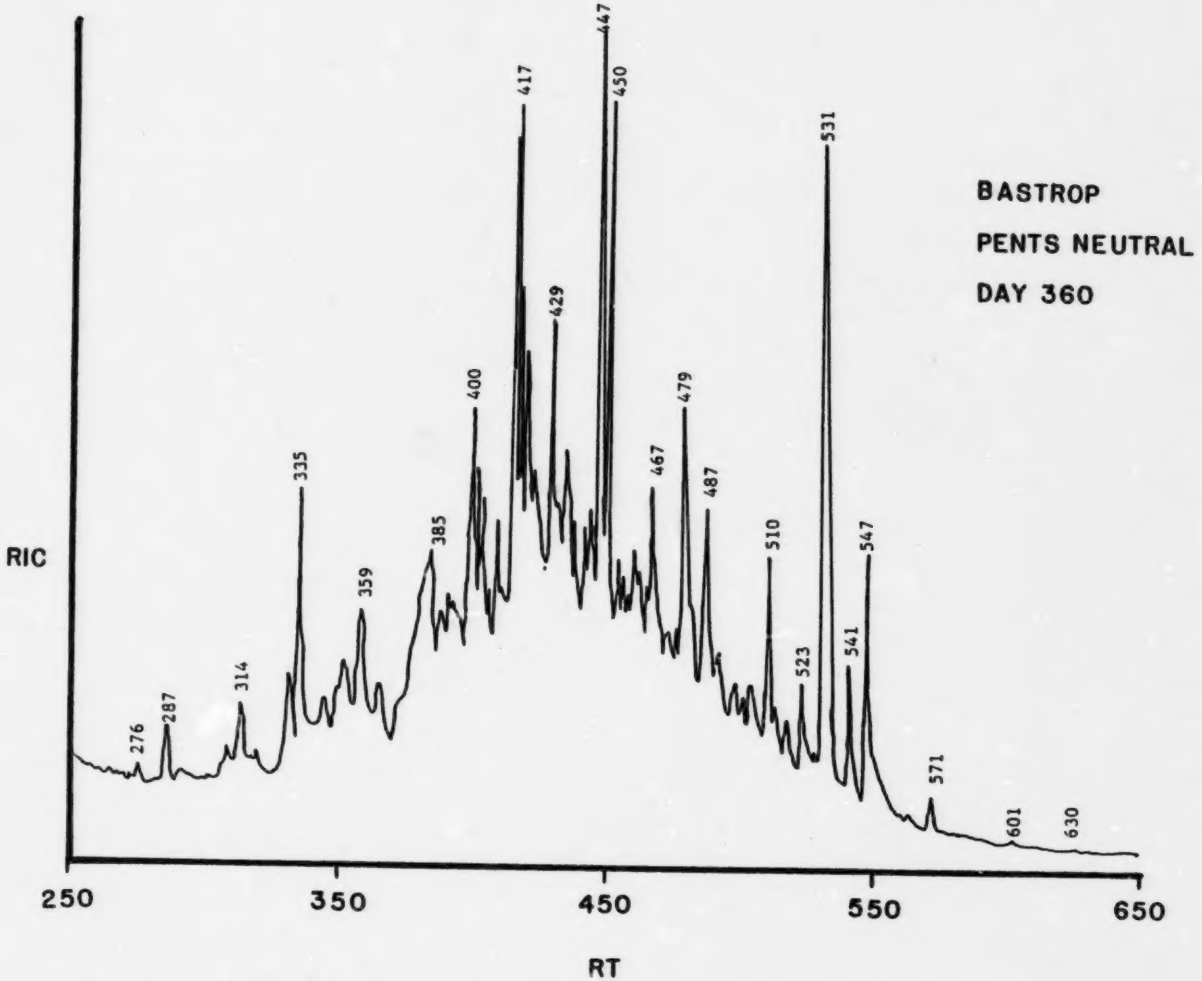

Figure 117. GC/MS chromatograph of neutral fraction of PENT S amended Bastrop soil collected on day 360.

Several polycyclic aromatic hydrocarbons (PAH) were also identified in the extracts of the PENT S amended soils. These included methylnapthalene, dimethyl napthalene, trimethyl napthalene, and dihydro acenapthylene. Of the methylated napthalenes, only methylnapthalene has been found to be mutagenic (Kaden et al., 1979). Although dihydro acenapthylene was not mutagenic in the standard reverse mutation assay (Gatehouse, 1980), it was positive when tested in the forward mutation assay (Kaden et al., 1979). Non-mutagenic PAH with three or more aromatic rings were identified in the soil-waste extracts and included anthracene, phenanthrene, and cyclopentaphenanthrene (McCann et al., 1975; Kaden et al., 1979). Of these compounds, only anthracene has been tested in a whole animal bioassay and was found to be non-carcinogenic (DHEW, 1969). Identified mutagenic PAH included methylphenanthrene, dimethyl phenanthrene (La Voie et al., 1983), and pyrene and fluoranthene (Kaden et al., 1979) which are not carcinogens (DHEW, 1969). Pyrene and fluoranthene have, however, displayed cocarcinogenic activity (Hoffman et al., 1982). A study by La Voie et al. (1981) found that methylphenanthrene and dimethylphenanthrene were mutagenic toward *Salmonella* and that only dimethylphenanthrene acted as a tumor initiator on mouse skin. Two polycyclic aromatic sulfur heterocycles were identified in the soil-waste extracts. Both of these compounds have been tested in the *Salmonella* assay, and dibenzothiophene and methyldibenzothiophene were not mutagenic (Pelroy et al., 1983). The only chlorinated hydrocarbon identified in the waste-soil extracts was pentachlorophenol. Pentachlorophenol is not mutagenic in the *Salmonella* assay (Anderson et al., 1972) but has been found to induce mitotic gene conversion in *S. cerevisae* (Fahrig, 1974). In addition, Schmid et al (1983) observed an increased incidence of chromosome damage in workers from a pentachlorophenol plant. Thus, chemical analysis of the PENT S amended soils identified more than fourteen polycyclic aromatic hydrocarbons, including four that were mutagenic and two animal carcinogens.

Although a large number of compounds were identified in the PENT S soil extracts, there were many additional compounds that were detected but not identified. These included a large number of higher molecular weight compounds that were possibly three to five ring PAH. The source of the waste and some of the potential soil metabolites can provide information as to the identity of these minor constituents. Technical grade pentachlorophenol is known to contain a number of impurities including tetrachlorophenol, dibenzo-p-dioxins, and dibenzofurans (Fishbein, 1979). Of these compounds, only dibenzofuran was detected in the waste-soil extracts from the greenhouse study. Creosote oil is composed of a variety of aromatic hydrocarbons and has been shown to be an indirect-acting mutagen (Bos et al., 1983). Since this oil was used in the process that generated the PENT S, the aromatic hydrocarbons which compose creosote oil would be anticipated in the soil-waste extracts. The biodegradation of some of the organic compounds present in waste fractions would be expected to produce metabolites such as hydroxylated napthalenes (Higgins and Gilbert, 1978) and catechol (Gibson, 1972). Catechol is not mutagenic for Salmonella but is a co-mutagen (Yoshida and Fukuhara, 1983) and a co-carcinogen (Van Duuren and Goldschmidt, 1976). Thus, the extracts of the PENT S amended soil contain a complex mixture of chemicals, including mutagens, carcinogens, teratogens, promotors, initiators, inhibitors, and co-carcinogens.

A comparison of the GC/MS chromatograms of the PENT S soil extracts from day 0 and 360 does not indicate any significant loss of organic compounds nor any appreciable gain in metabolic products from soil degradation (Figures 107 to 117). Methylnapthalene was the only mutagenic compound present in day 0 samples that was not also identified in the soil from day 360. Potential biodegradation products that were identified in the soil extracts from day 360 include methyl propyl pentanol, ethyl methyl pentanol, and ethyl hexanol. The enzymes responsible for the formation of these compounds are mixed function oxygenases (McKenna, 1972). If these compounds are products of soil degradation, these results indicate that the enzymes responsible for activating promutagens may be present in the soil. Thus, the increased activity observed in some of the incubated soil samples was possibly a result of soil activation or a result of decreased competition for the activating enzymes used in the mutagenesis bioassays.

The GC/MS analysis was unable to conclusively identify any of the components of the acid, base, and neutral fractions of the SWRI waste or the day 0 extracts. Additional concentration of the day 360 soil samples allowed the identification of seven compounds and the detection of 25 additional unknown compounds. The compounds identified in the day 360 extract from the SWRI waste amended Bastrop soil included heptadecane, nonadecane, dihydro acenapthylene, dimethyl octane, ethyl propyl hexanol, trimethyl decane, and pyrene (Figures 118, 119 and 120). Only two of these compounds, dihydro acenapthylene in the base and pyrene in the neutral fraction, are potential sources of mutagenic activity (Kaden et al., 1979). Kaden et al. (1979) indicate that the mutagenic potential of acenapthylene and pyrene are approximately equal. However, biological analysis demonstrated that the mutagenic potential of the base fraction was three to four times that of the neutral fraction. An evaluation of the combined results from the chemical and biological analysis of the day 360 samples from the SWRI waste amended Bastrop soil indicate that the decreased mutagenic activity observed in the neutral fraction may have resulted from inhibition produced by the non-mutagenic components of the neutral fraction.

A list of the compounds detected in the COMBO waste amended soils is provided in Table 92, and the GC/MS chromatograms are given in Figures 121 through to 131. Unlike the PENT S waste, a review of the GC/MS chromatograms from the COMBO waste amended soil does indicate that significant quantities of organic compounds are degraded in the soil. The GC/MS chromatograms of the acid fraction of the Norwood soil

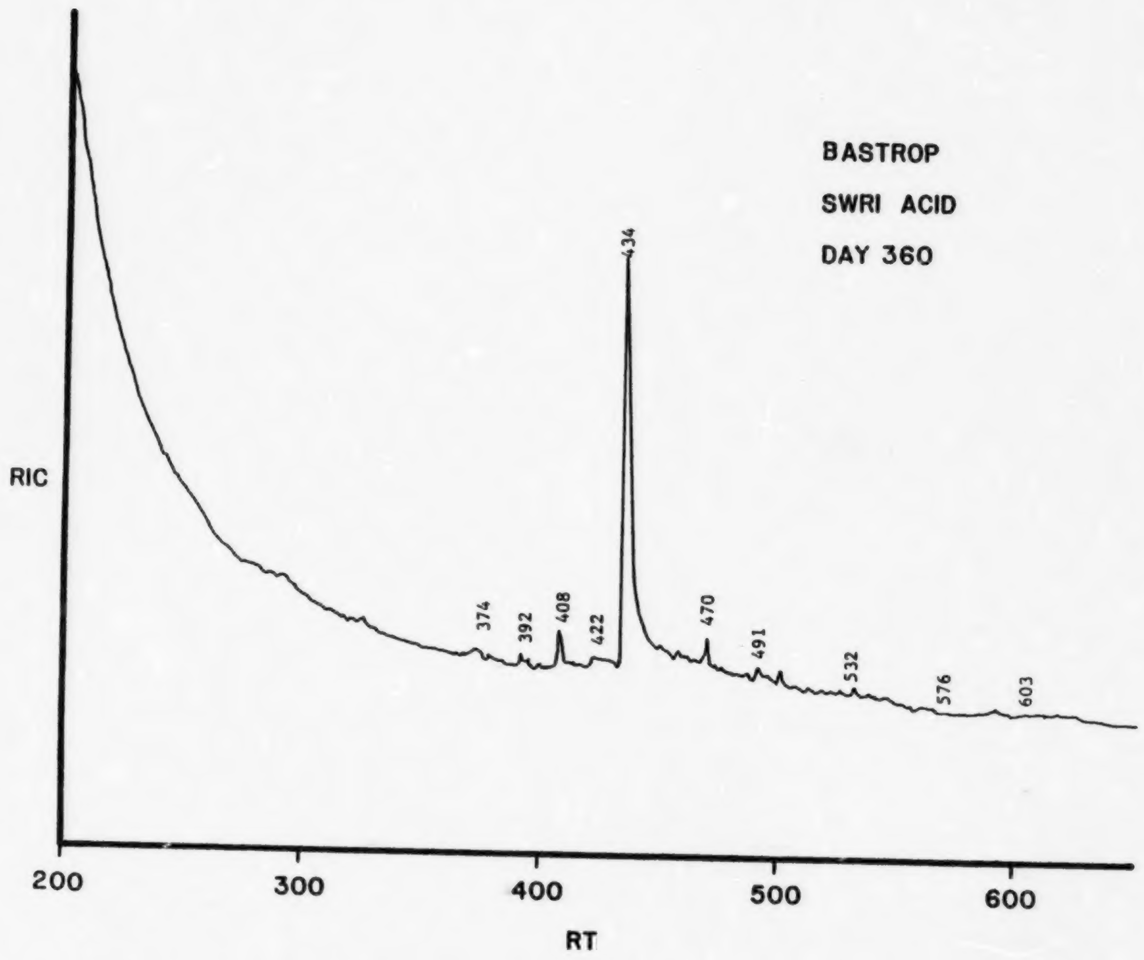

Figure 118. GC/MS chromatograph of acid fraction of SWRI amended Bastrop soil collected on day 360.

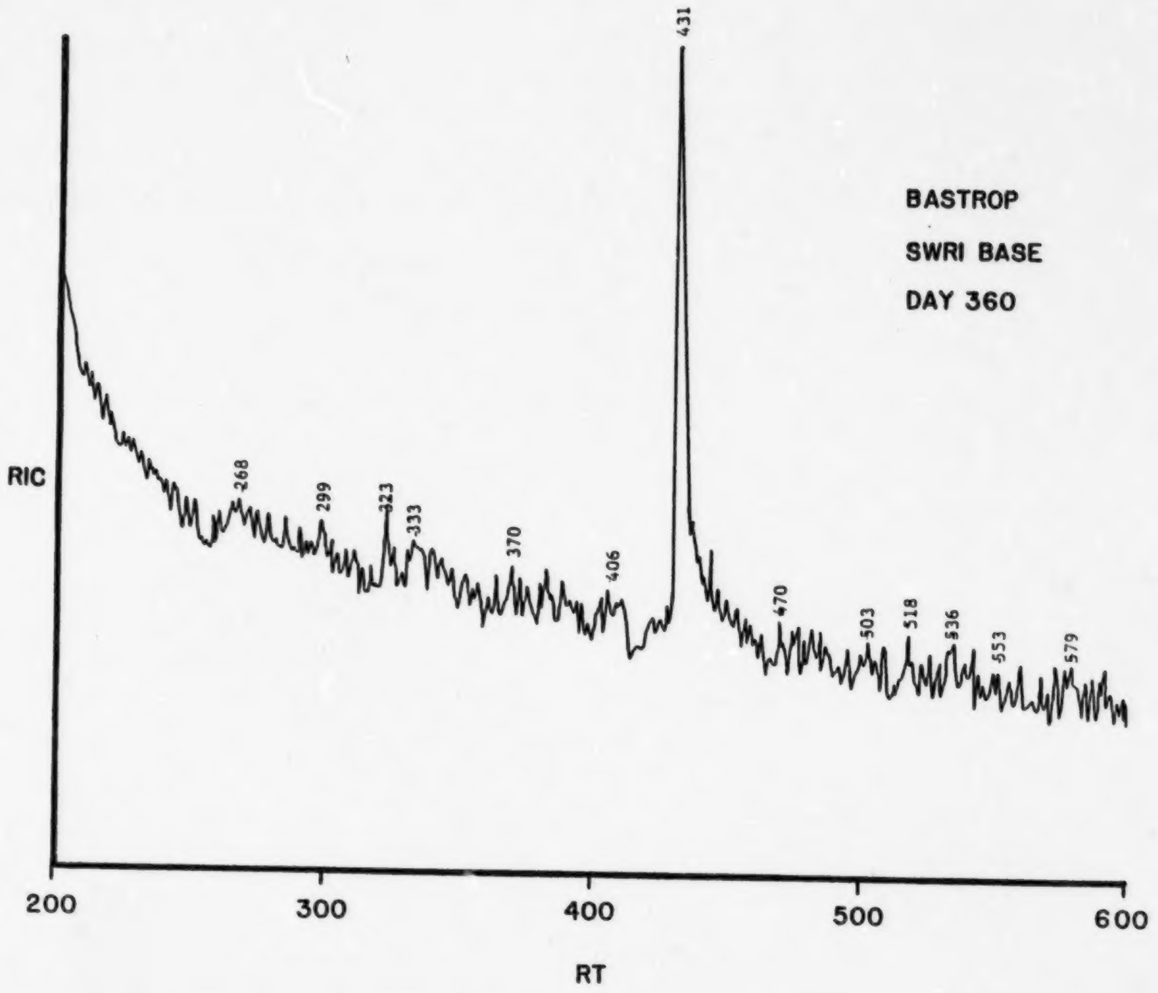

Figure 119. GC/MS chromatograph of base fraction of SWRI amended Bastrop soil collected on day 360.

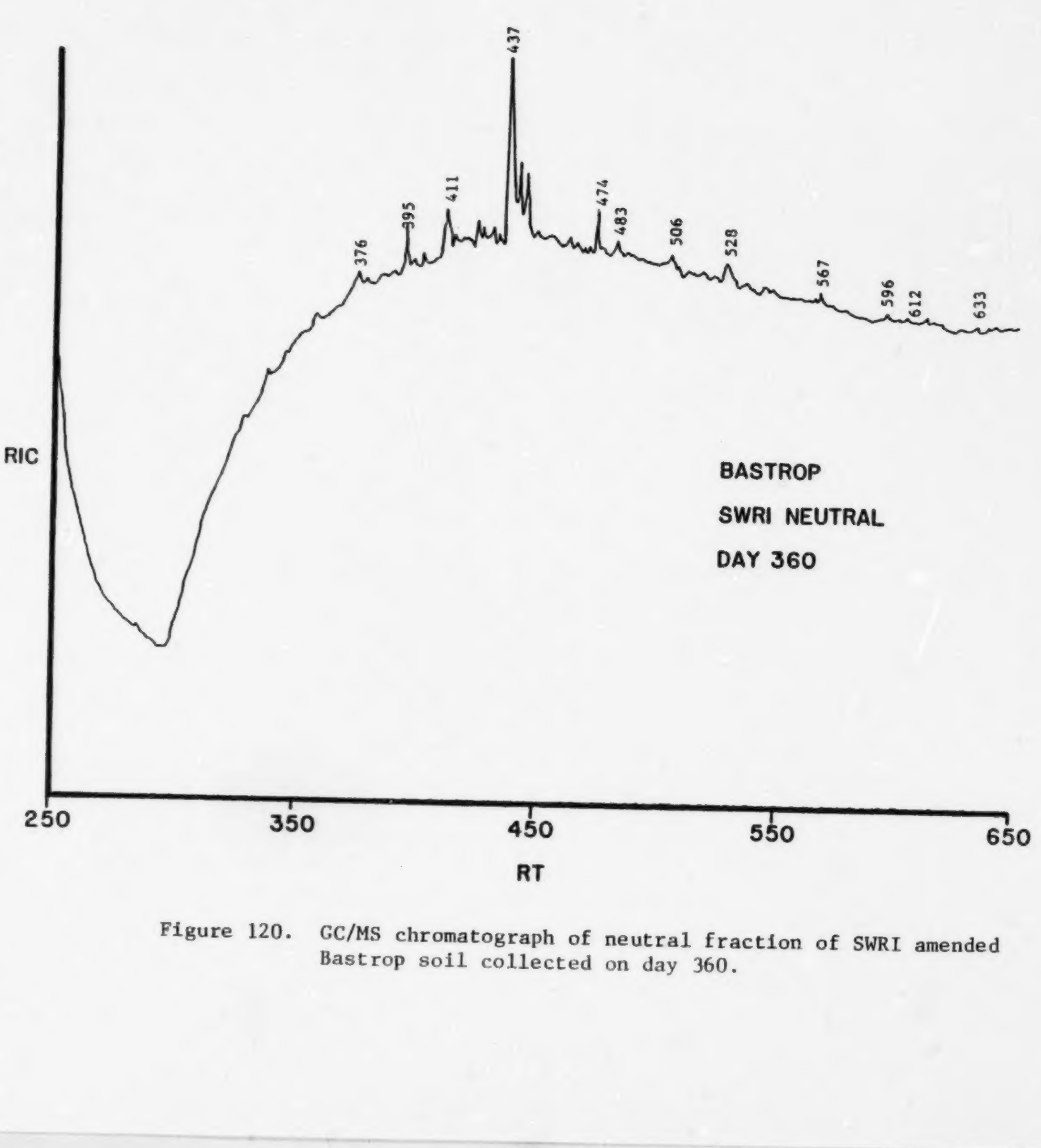

Figure 120. GC/MS chromatograph of neutral fraction of SWRI amended Bastrop soil collected on day 360.

TABLE 92. LIST OF COMPOUNDS DETECTED IN COMBO WASTE AMENDED SOIL

Sample	Fraction		
	Acid Compound (Peak Number)*	Base Compound (Peak Number)*	Neutral Compound (Peak Number)*
Norwood Day 0	Unknown (226)	Dimethyl decane (272)	Unknown (227)
	Trimethyl octane (233)	Dimethyl undecane (300)	Methyl napthalene (251)*MI; CO
	Unknown (245)	Unknown (367)	Unknown (258)
	Phenyl cyclopentanol (257)	Unknown (385)	Trimethyl octane (273)
	Methyl napthalene (263)*MI; CO	Unknown (406)	Dimethyl napthalene (288) (299)*MO
	Trimethyl dodecane (280)	Unknown (437)	Unknown (303)
	Unknown (295)	Unknown (470)	Unknown (322)
	Dimethyl napthalene (300)(305)(313)*MO	Unknown (517)	Methylethyl napthalene (329)
	Propyl heptanol (318)	Unknown (549)	Trimethyl napthalene (339) (347) (352)*MO
	Methyl ethyl napthalene (339)	Unknown (583)	Propylmethyl napthalene (367)
	Trimethyl napthalene (350)(356)(364)*MO	Unknown (609)	Unknown (375)
	Methyl propyl napthalene (377)	Unknown (643)	Methyl ethyl decane (386)
	Unknown (386)	Unknown (675)	Unknown (395)
	Undecane (396)	Unknown (708)	Methyl fluorene (408)
	Methyl (Meth) napthalene (405)		Unknown (446)
	Ethyl tridecane (418)		Methyl phenanthrene (480)*MI
	Dimethyl undecane (457)		Unknown (505)
	Unknown (477) (492)		Dimethyl phenanthrene (519)*MI; CI
Norwood Day 360	Unknown (335)	Tridecane (236)	Unknown (330)
	Unknown (357)	Unknown (258)	Unknown (353)
	Unknown (376)	Tetradecane (279)*P	Unknown (353)
	Methyl decane (397)	Dimethyl napthalene (295)*MO	Unknown Branched Alkane (399)
	Unknown (468)	Unknown (308)	Dimethyl nonane (415)
	Unknown (492)	Pentadecane (319)	Unknown (429)
	Unknown (527)	Unknown (331)	Unknown (468)
	Unknown (548)	Fluorene (355)*MO; CO	Dimethyl decane (479)
	Unknown (576)	Hexadecane (360)*A	Unknown (489)
		Dimethyl undecane (379)	Unknown (510)
		Heptadecane (399)	Unknown (533)
		Phenanthrene (428)*MO; CO	Unknown (572)
		Octadecane (436)*P; CC	
		Unknown (456)	
		Nonadecane (471)	
		Benzene dicarboxylic acid (493)*MO; CO	
		Eicosane (505)*CC	
		Fluoranthene (525)*MI; CO	

TABLE 92 CONTINUED

Sample	Fraction Acid Compound (Peak Number)*	 Base Compound (Peak Number)*	 Neutral Compound (Peak Number)*
Norwood Day 360		Heneicosane (536) Pyrene (541)*MI; CO Docasane (566) Unknown (577)(596)	
Bastrop Day 0	Unknown (224) Methylnapthalene (249)(255)*MI; CO Alkane (272) Dimethyl napthalene (291)(297)(305)*MO Dihydro acenaphthylene (323)*MI Methyl ethyl napthalene (331) Trimethyl napthalene (341)(349)(355)*MO Propenyl napthalene (369) Unknown (377) (388) (396) Methyl fluorene (409 Dimethyl fluorene (453) Methyl phenanthrene (481)*MI Unknown (507) (527) (606) (641) (683)	Methyl napthalene (253)*MI Dimethyl napthalene (295)*MO Ethyl napthalene (303) Alkane (309) Methyl ethyl napthalene (328) Trimethyl napthalene (338)(344) (351)*MO Methyl propyl napthalene (364) Unknown (405) (479) (513) (555) (592) (657) (697) (755) (795) (838) (877)	Methyl napthalene (245)*MI Alkane (271) Dimethyl napthalene (285)(291)*MO Propyl heptanol (303 Methyl ethyl napthalene (327) Trimethyl napthalene (338)(346)(352)*MO Propenyl napthalene (367) Unknown (387) Dimethyl biphenyl (408) Methyl phenanthrene (481)*MI Unknown (526) (600) (689) (736) (801) (847)
Bastrop Day 360	Unknown (327) Dimethyl napthalene (341)*MO Dihydro acenapthylene (356)*MI Phenalene (380)*MI Unknown (395) Dimethyl undecane (407) Unknown (457) Trimethyl decane (467)(498) Unknown (476) (520) (536) (558) (587)	Not determined	Unknown (289) Benzene dicarboxylic acid (484)*MO;CO Unknown (543)

* Potential genetic toxicity: I = initiator; P = promotor; A = antagonist; CC = cocarcinogen(synergist); MO = nonmutagenic; MI = mutagenic; CO = non-carcinogen; Cl = carcinogen; all others = no information. The references used in determining these factors McCann et al (1975); Kaden et al (1979); Goldschmidt (1981) and DHEW (1969).

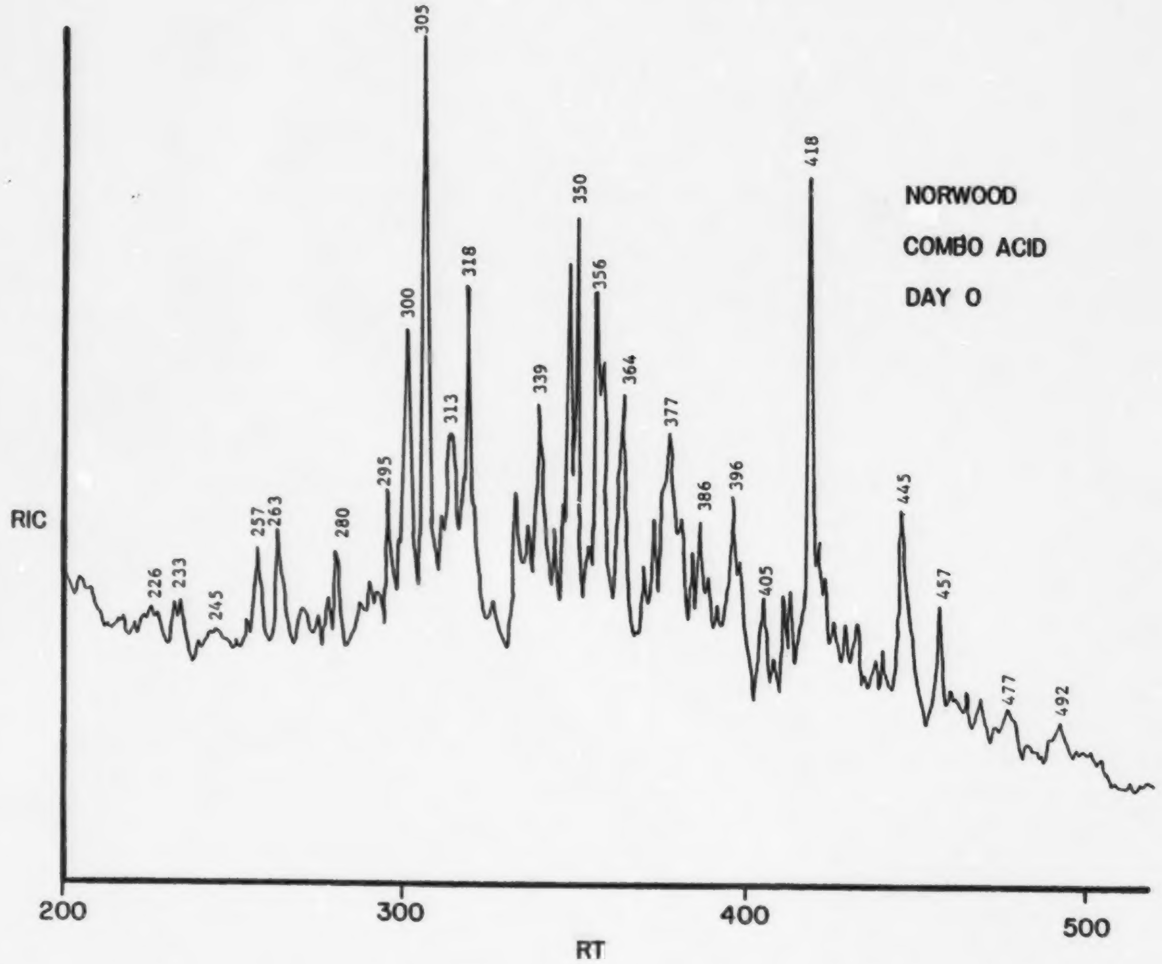

Figure 121. GC/MS chromatograph of acid fraction of COMBO amended Norwood soil collected on day 0.

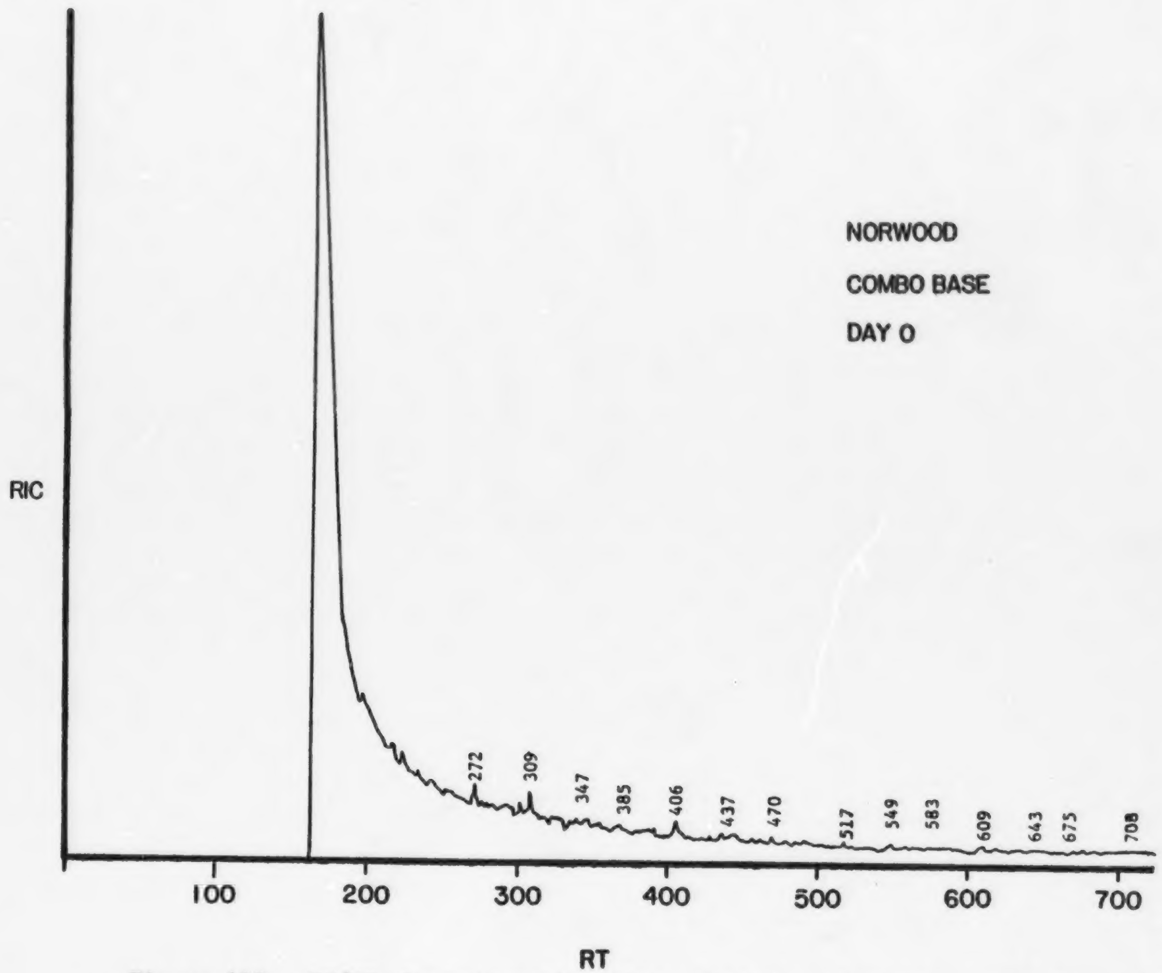

Figure 122. GC/MS chromatograph of base fraction of COMBO amended Norwood soil collected on day 0.

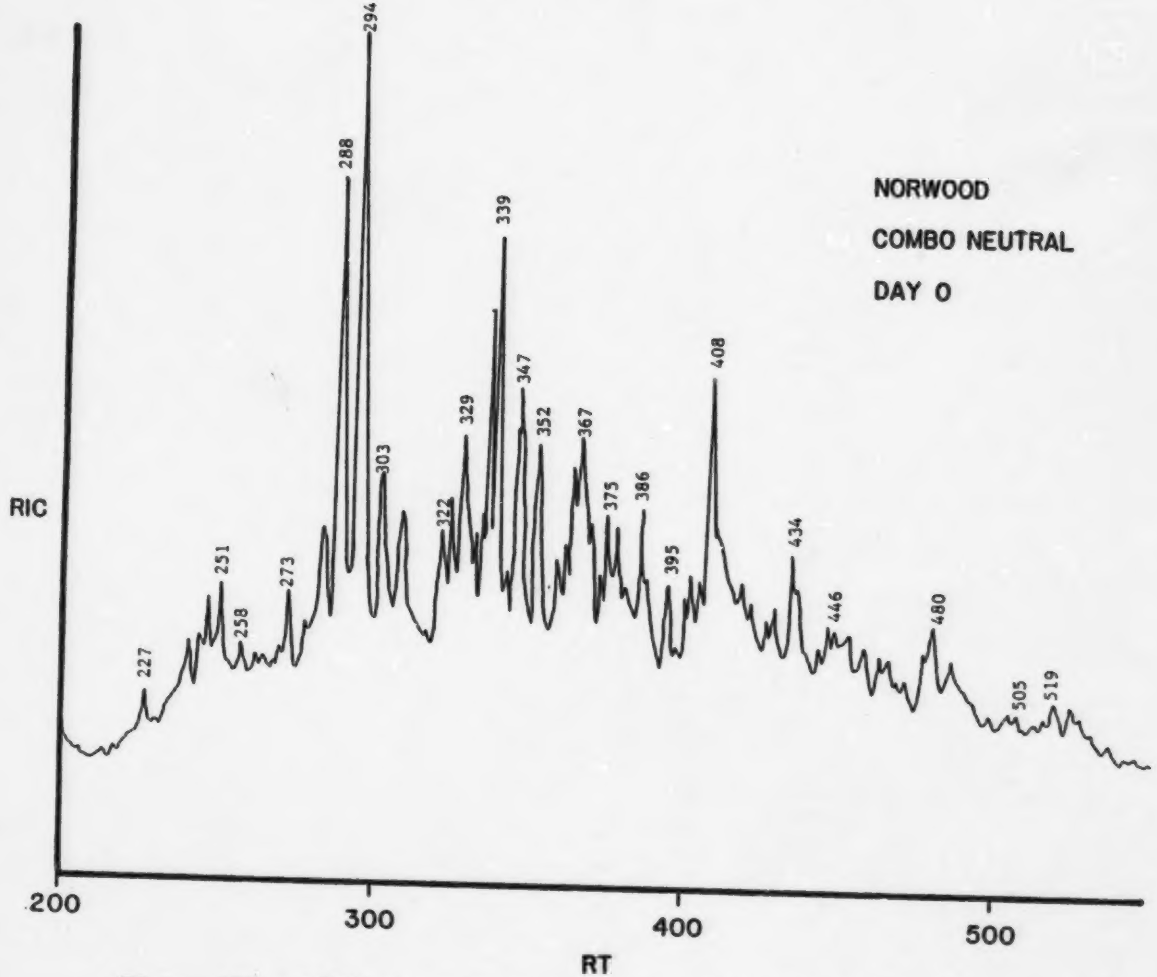

Figure 123. GC/MS chromatograph of neutral fraction of COMBO amended Norwood soil collected on day 0.

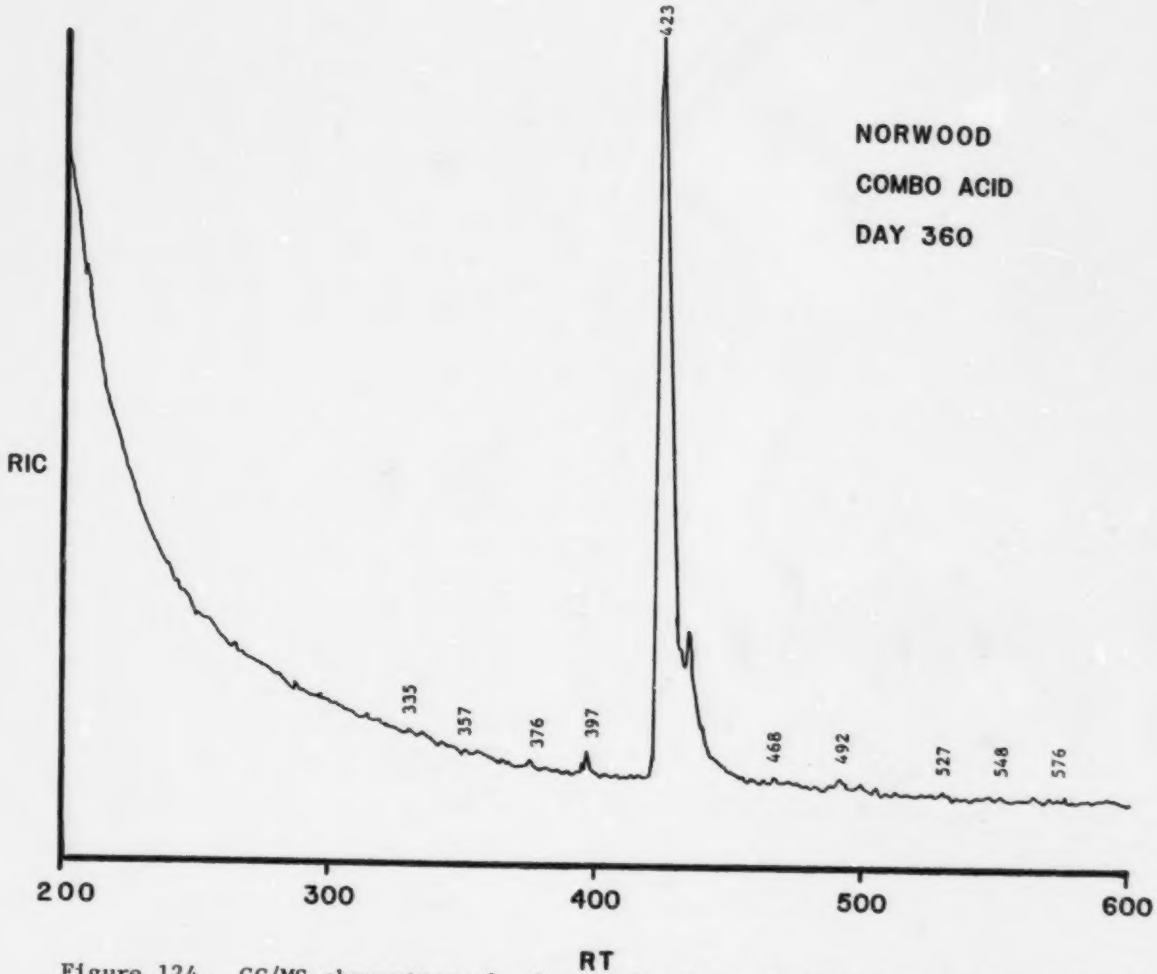

Figure 124. GC/MS chromatograph of acid fraction of COMBO amended Norwood soil collected on day 360.

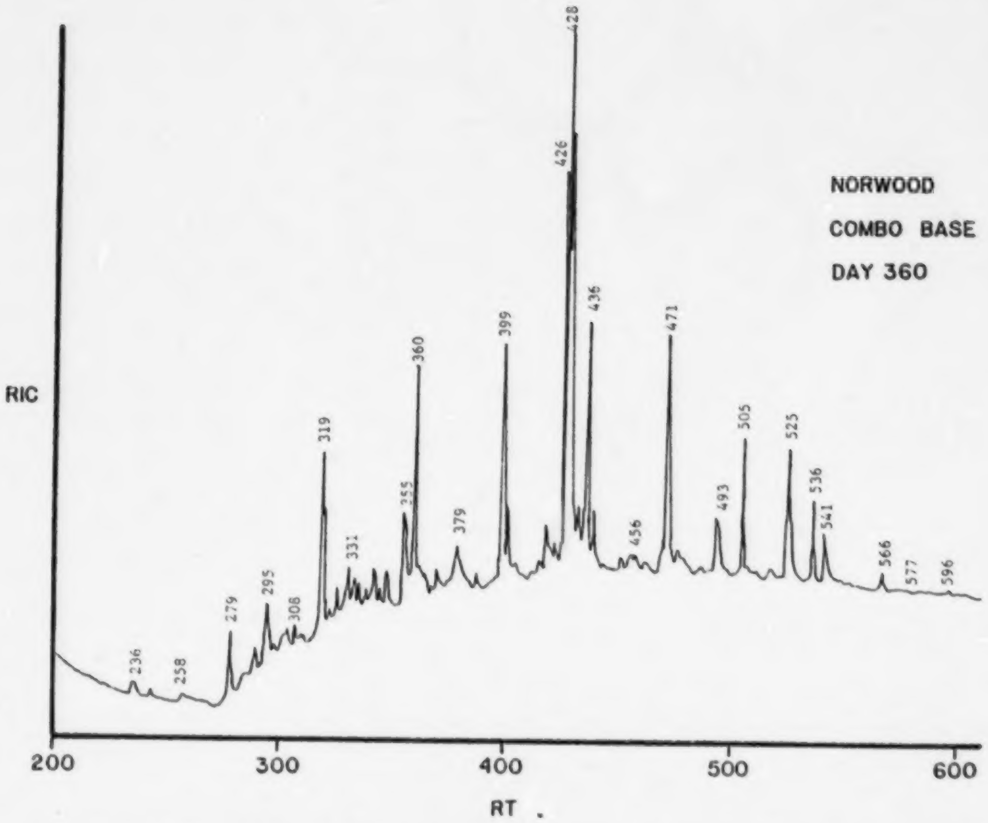

Figure 125. GC/MS chromatograph of base fraction of COMBO amended Norwood soil collected on day 360.

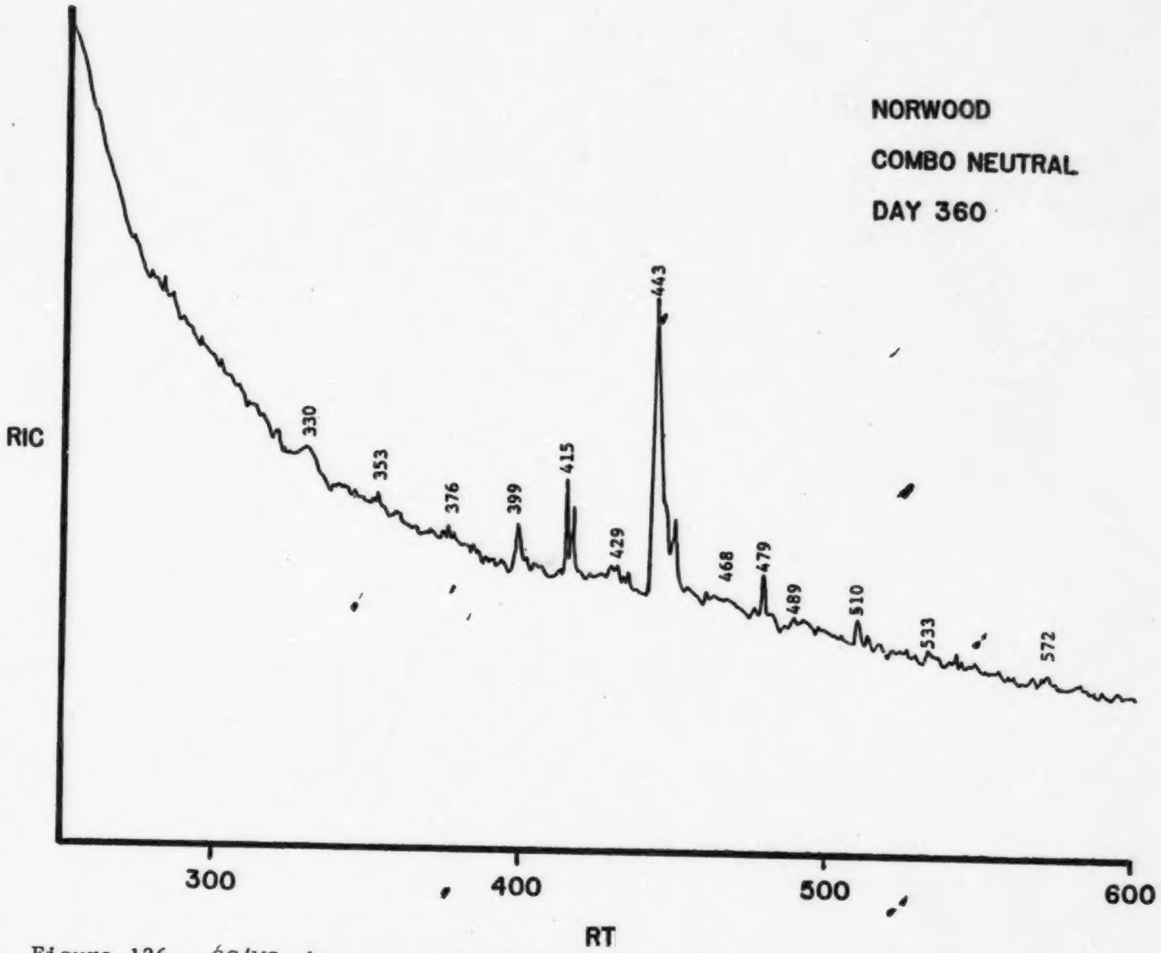

Figure 126. GC/MS chromatograph of neutral fraction of COMBO amended Norwood soil collected on day 360.

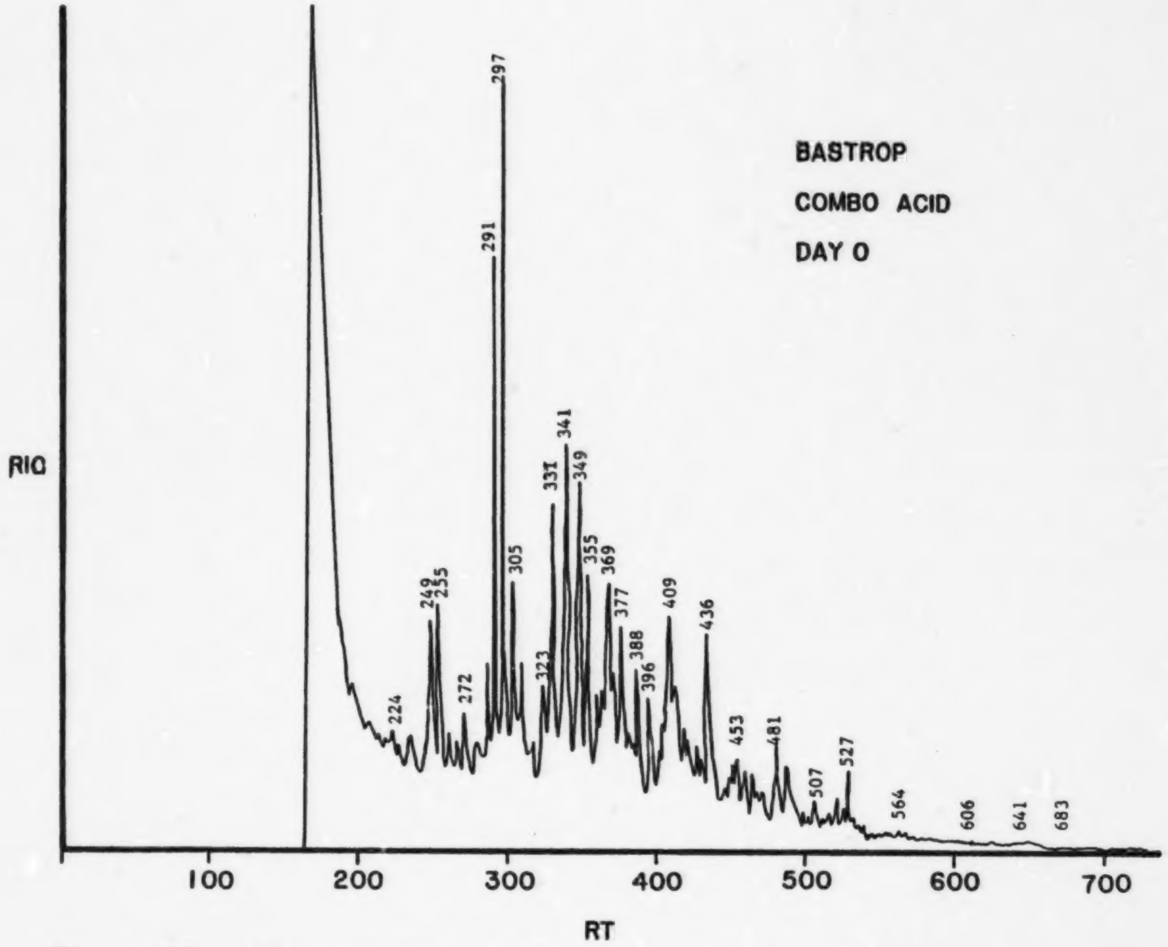

Figure 127. GC/MS chromatograph of acid fraction of COMBO amended Bastrop soil collected on day 0.

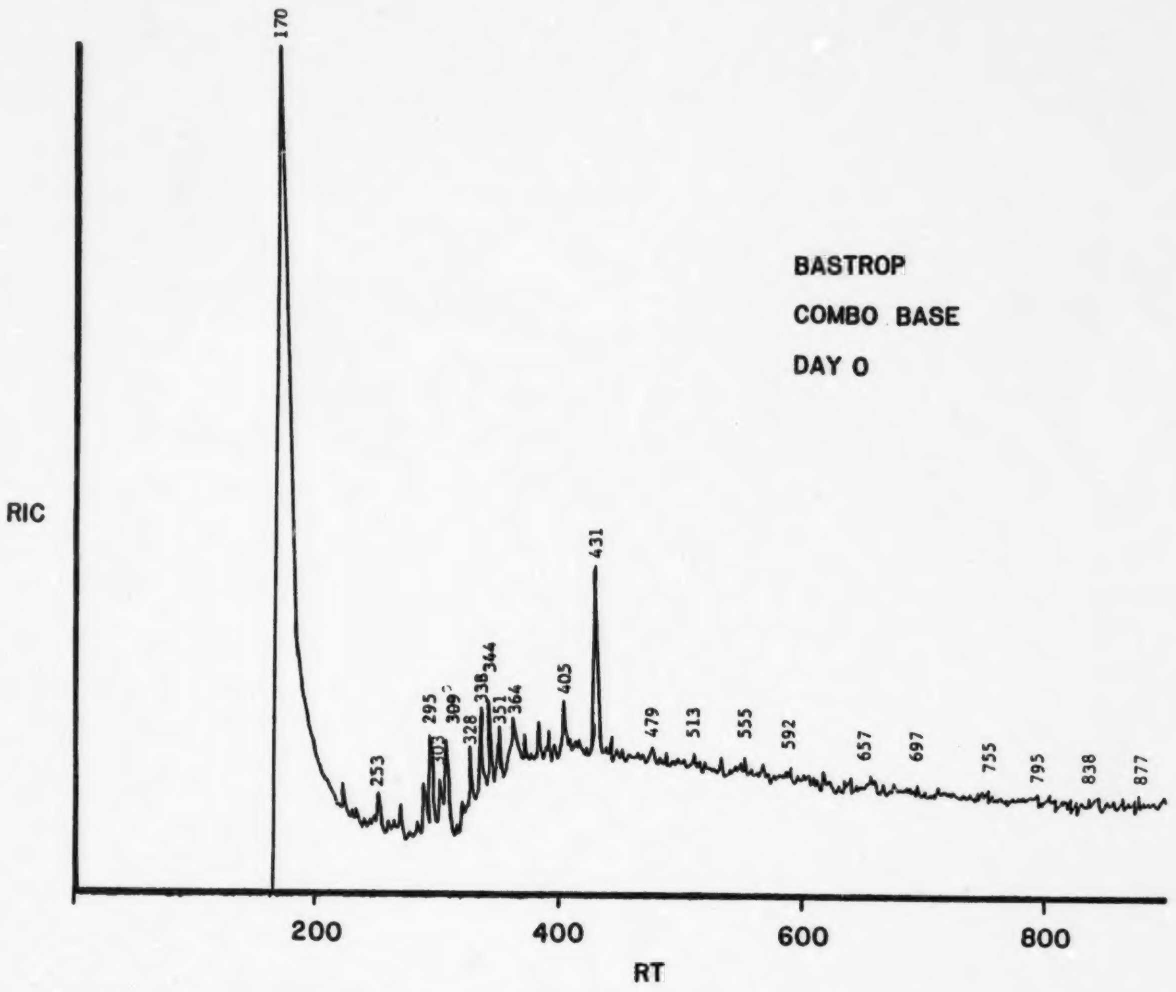

Figure 128. GC/MS chromatograph of base fraction of COMBO amended Bastrop soil collected on day 0.

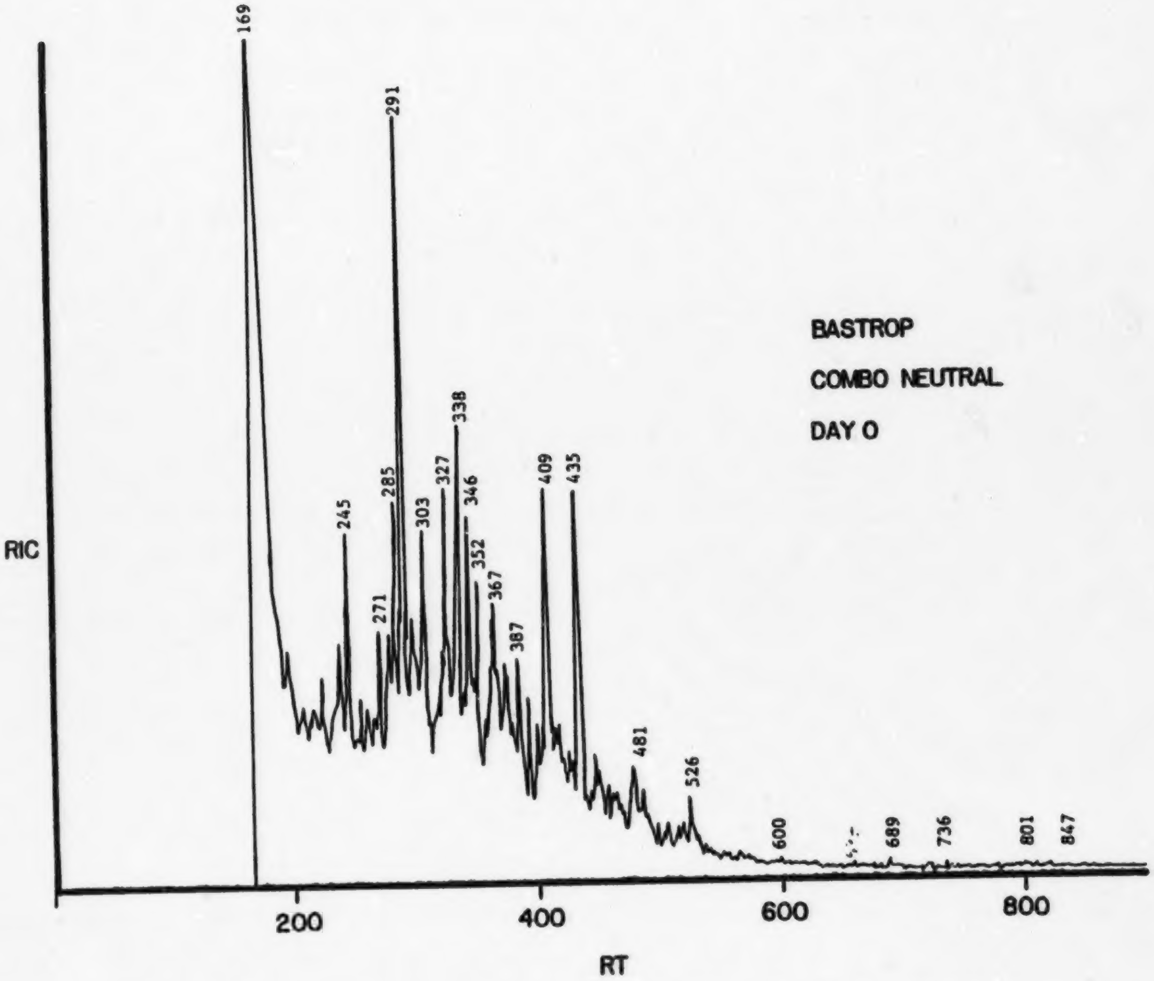

Figure 129. GC/MS chromatograph of neutral fraction of COMBO amended Bastrop soil collected on day 0.

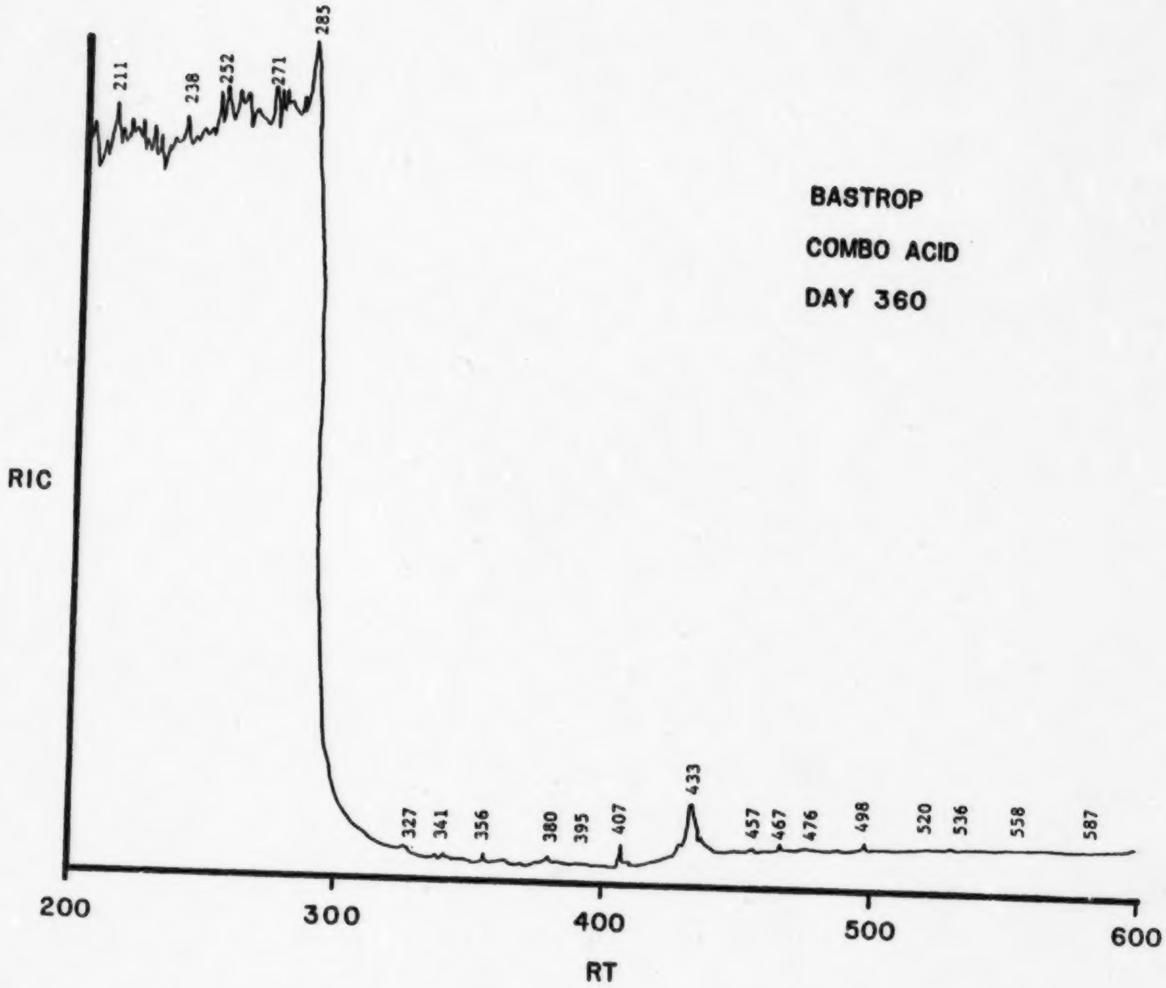

Figure 130. GC/MS chromatograph of acid fraction of COMBO amended Bastrop soil collected on day 360.

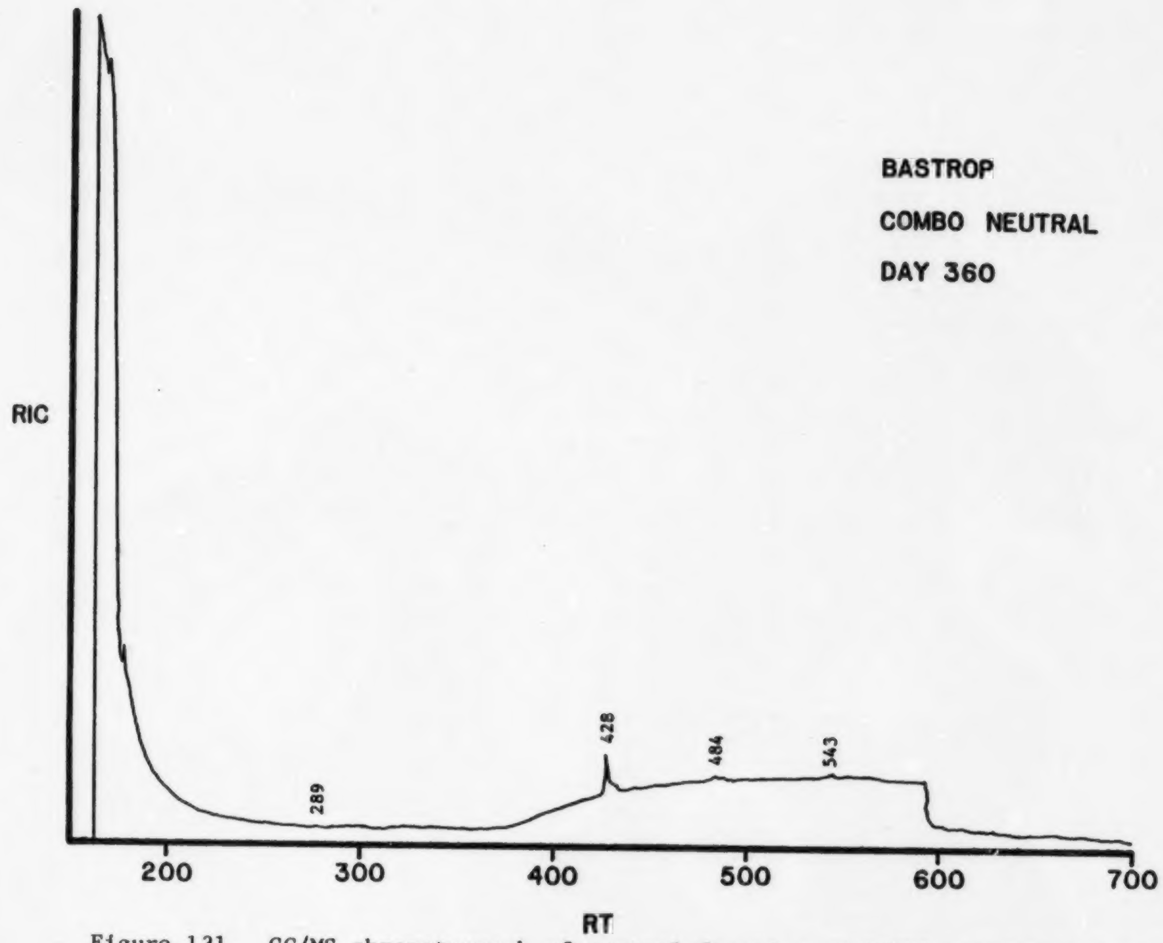

Figure 131. GC/MS chromatograph of neutral fraction of COMBO amended Bastrop soil collected on day 360.

indicates that on day 0 there were twelve compounds identified with an additional six unknown compounds detected (Figure 118). Of the twelve compounds identified, only methylnapthalene is mutagenic (Kaden, 1979). The acid fraction from day 360 contained only one identifiable and eight unknown detectable compounds (Figure 118). However, the bioassay of the acid fractions from day 0 and 360 did not reflect a reduced level of mutagenic activity (Table 85). Similar results were obtained from the neutral fraction from day 0 with ten compounds identified plus eight unknown detected (Figure 119). While three of the ten compounds identified in the day 0 sample were mutagenic, none of the mutagens were also detected in the extract from day 360 (Table 90). The reverse trend seemed to occur in the base fraction with the GC/MS chromatograph from day 360 (Figure 125) appearing more complex than the sample from day 0 (Figure 122). The base fraction from COMBO waste amended soils also showed a corresponding increase in mutagenic activity on day 360. (Figures 101 and 104). The results from chemical and biological analysis of the acid fraction of the COMBO waste amended soils appear to contradict each other, while the chemical analysis of the base and neutral fractions provides confirmation of the respective increased and decreased mutagenic activity that was observed in the bioassays.

Neither chemical analysis of the soil extracts nor biological analysis of residual hydrocarbons have been capable of clearly demonstrating treatment of soil applied waste. The results already presented in Figures 83 through 106 describe the mutagenicity of the residual hydrocarbons in waste amended soil. The results presented in Figures 107 through 131 identify the major organic constituents residual in the soil. However, in order to determine if a waste is rendered less or non-hazardous by soil incorporation, it is necessary to compare the mutagenic potential of equal volumes of waste-amended soil. In the Salmonella assay, the mutagenic potential (Figures 132 to 134) was determined by calculating the mutagenic activity ratio (Commoner, 1976) of two non-toxic dose levels from a five member dose-response curve (Table 73 through 90 and Figures 83 through 106) and adjusting this ratio by the rate of hydrocarbon degradation. While in the Aspergillus methionine assay (Figures 135, 136 and 137) equivalent volumes of soil were compared by evaluating the total mutation frequency induced by the soil-waste extract. The concentration of extract tested in each assay was adjusted according to the rate of degradation. Thus, the data presented in Figures 132 through 137 is not an accurate representation of the mutagenic activity of soil-waste extracts. The utility of these data is their ability to define the hazardous characteristics of equal volumes of waste-amended soil. The volume tested from the acid and base fractions of the SWRI and COMBO waste-amended soils was the equivalent of 1 gram. However, the neutral fraction from the SWRI and COMBO soils, and all three fractions from the PENT S soils were tested in the Aspergillus assay at a level twice that tested in the Salmonella assay. While it is unfortunate that equal volumes were not tested in all assays, these results do allow some general comparisons to be made. By comparing the mutagenic potential of equivalent volumes of waste amended soil over time, it is possible to determine if a waste is rendered less or non-hazardous by soil incorporation.

The results from evaluating the affect of soil degradation on the mutagenic potential of PENT S amended soils are presented in Figures 132 and 135. In the Norwood soil, the mutagenic potential of the base and neutral fractions decreased to below the significant level (two times solvent control), in both bioassays. In the acid fraction, the mutagenic potential with metabolic activation was reduced to below a level at which the sample would be considered mutagenic in both bioassays, although the response without activation in Aspergillus was increased by more than 50% from day 0 to 360. The mutagenic potential of the neutral fraction from PENT S amended Bastrop soil was also decreased to below significant levels in both bioassays. In the acid and base fractions from PENT S amended Bastrop soil, the mutagenic potential was increased on day 180. The responses in Aspergillus from both fractions were reduced by day 360 to less than that observed on day 0; and in Salmonella the mutagenic potential of the base fraction was reduced by day 360 while the response from the acid fraction did not decrease until day 540. Thus, both bioassays detected constituent(s) of the acid fraction of the PENT S waste amended soil that were resistant to degradation and highly mutagenic. The bioassays also indicated that the bulk of the total extractable hydrocarbons were rendered non-hazardous by land treatment.

The mutagenic potentials of the extracts of SWRI amended soils are presented in Figures 133 and 136. In the Norwood soil, the mutagenic potential of the acid and base fractions was not reduced to below the significant level in either of the bioassays 360 days after waste application. Although there was an appreciable reduction of the mutagenic potential of these two fractions in the Aspergillus assay, the mutagenic potential of the acid and base fractions on day 360, as measured with S. typhimurium, was approximately twice the mutagenic potential of an equivalent volume of soil from day 0. In both bioassays, the mutagenic potential of the neutral fraction from day 360 was approximately 25% that of the sample from day 0. The acid fraction of the SWRI waste amended Bastrop soils induced a lower response on day 360 than on day 0; however, in both bioassays the response induced by the sample collected on day 360 would be considered mutagenic. The response induced by the base fraction from SWRI waste amended Bastrop soils increased from day 180 to 360 in both the Salmonella and Aspergillus assays. Although the response induced by the sample from day 360 was below a level at which a sample would be considered mutagenic in Salmonella, in Aspergillus the response from the base fraction collected on day 360 was slightly greater than this level. The response induced by the neutral fraction extracted from the SWRI waste amended Bastrop soil collected on day 360 was less than the significant level in both bioassays. A comparison of the results from the two bioassays indicates that degradation did not render the acid and base

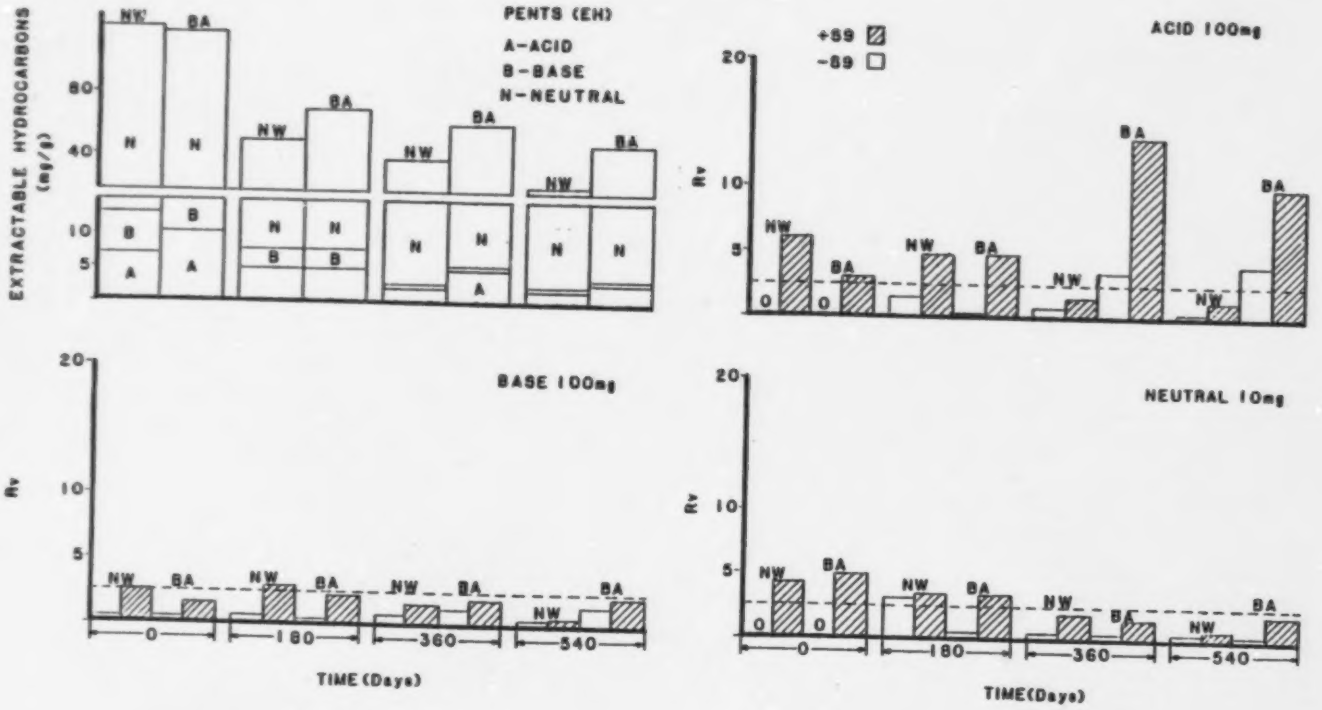

Figure 132. Total extractable hydrocarbons and mutagenic potential of equivalent volumes of PENT S amended Norwood (NW) and Bastrop (BA) soils as measured with S. typhimurium strain TA98 with and without metabolic activation. Dashed line (---) is equal to 2.5 times solvent control.

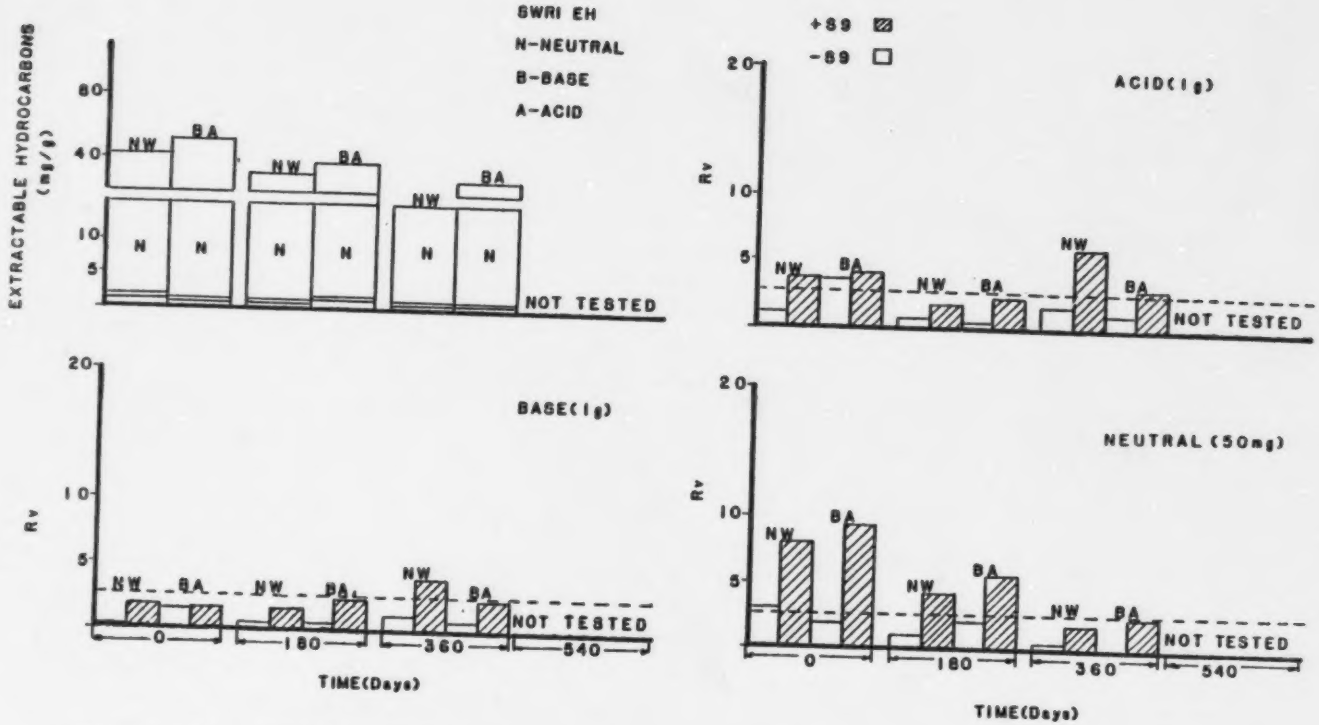

Figure 133. Total extractable hydrocarbons and mutagenic potential of equivalent volumes of SWRI amended Norwood (NW) and Bastrop (BA) soils as measured with S. typhimurium strain TA98 with and without metabolic activation. Dashed line (---) is equal to 2.5 times solvent control.

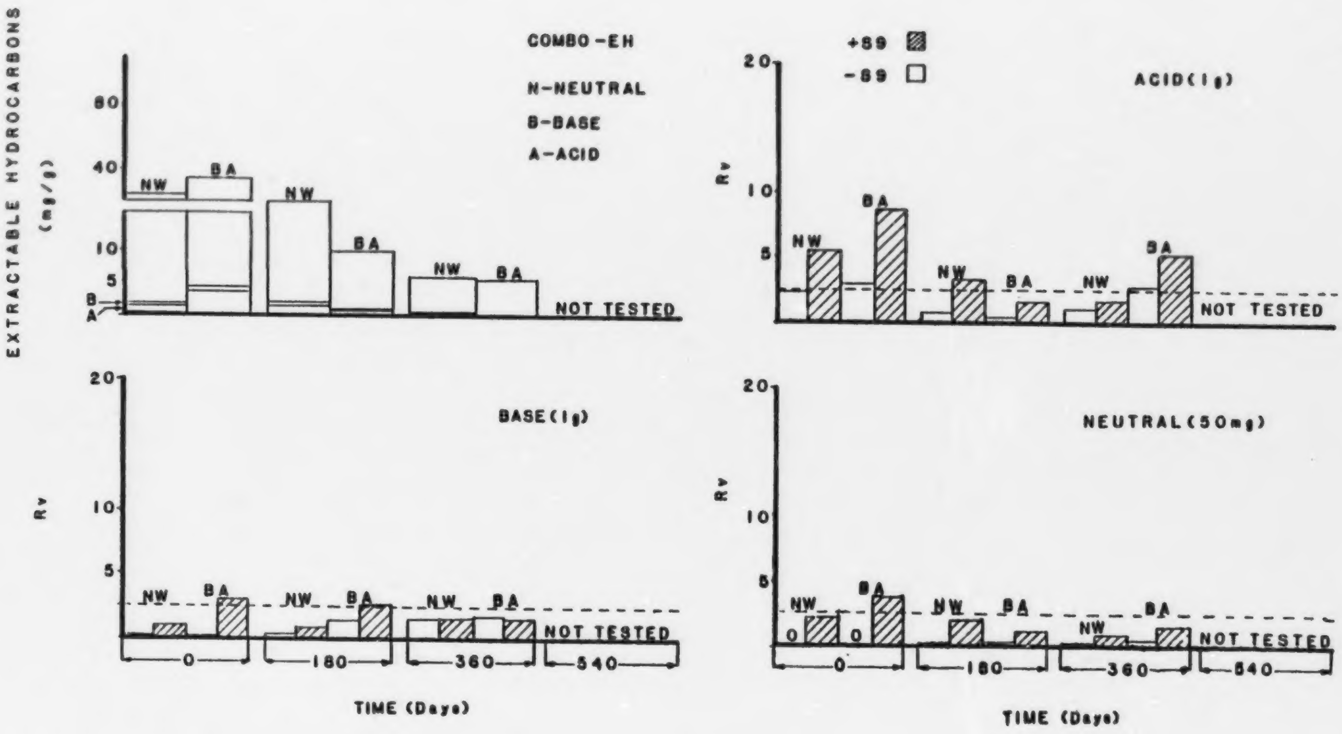

Figure 134. Total extractable hydrocarbons and mutagenic potential of equivalent volumes of COMBO amended Norwood (NW) and Bastrop (BA) soils as measured with S. typhimurium strain TA98 with and without metabolic activation. Dashed line (---) is equal to 2.5 times solvent control.

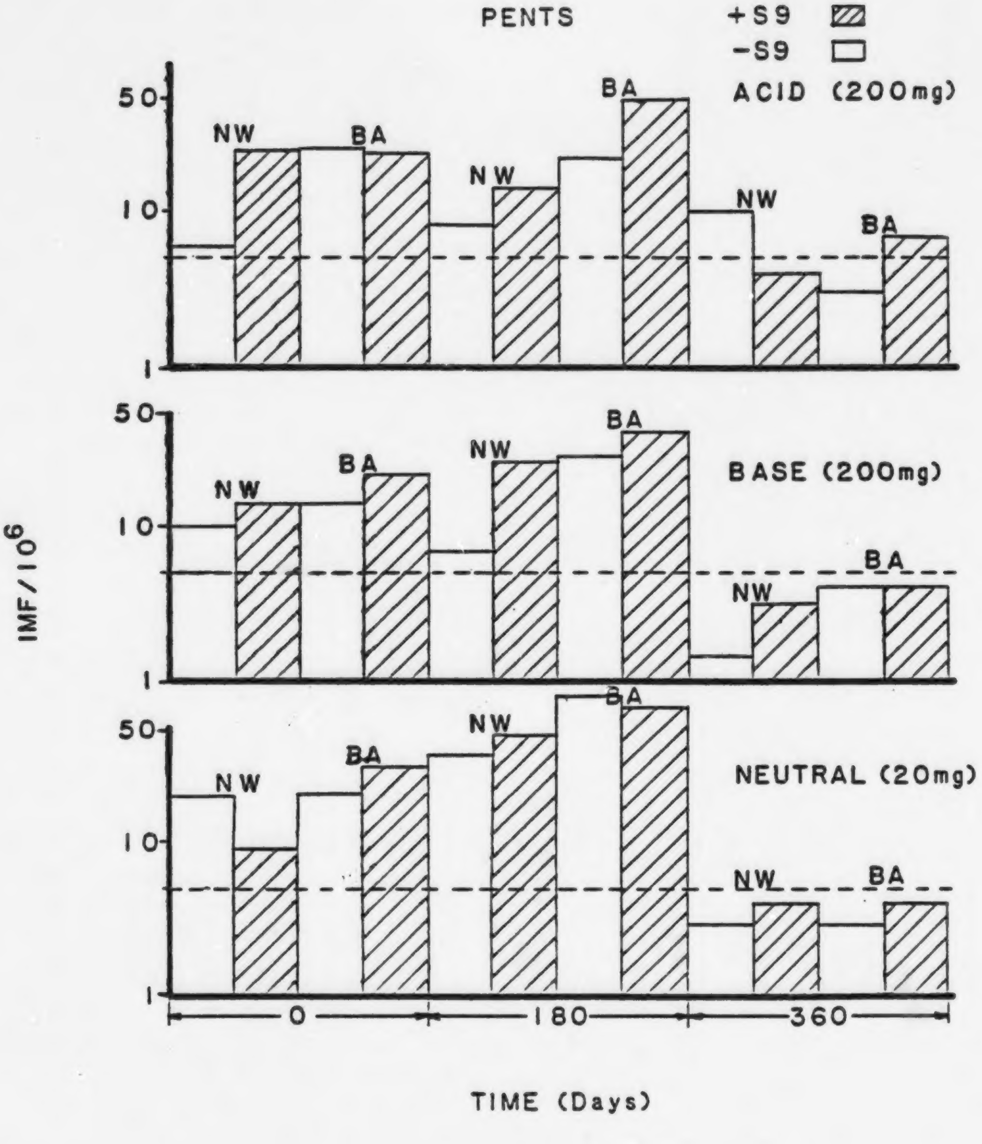

Figure 135. Total induced mutation frequency of equivalent volumes of PENT S amended Norwood (NW) and Bastrop (BA) soils as measured in A. nidulans methionine system with and without metabolic activation. Dashed line (---) is equal to total induced mutation frequency of $5.0/10^6$ survivors.

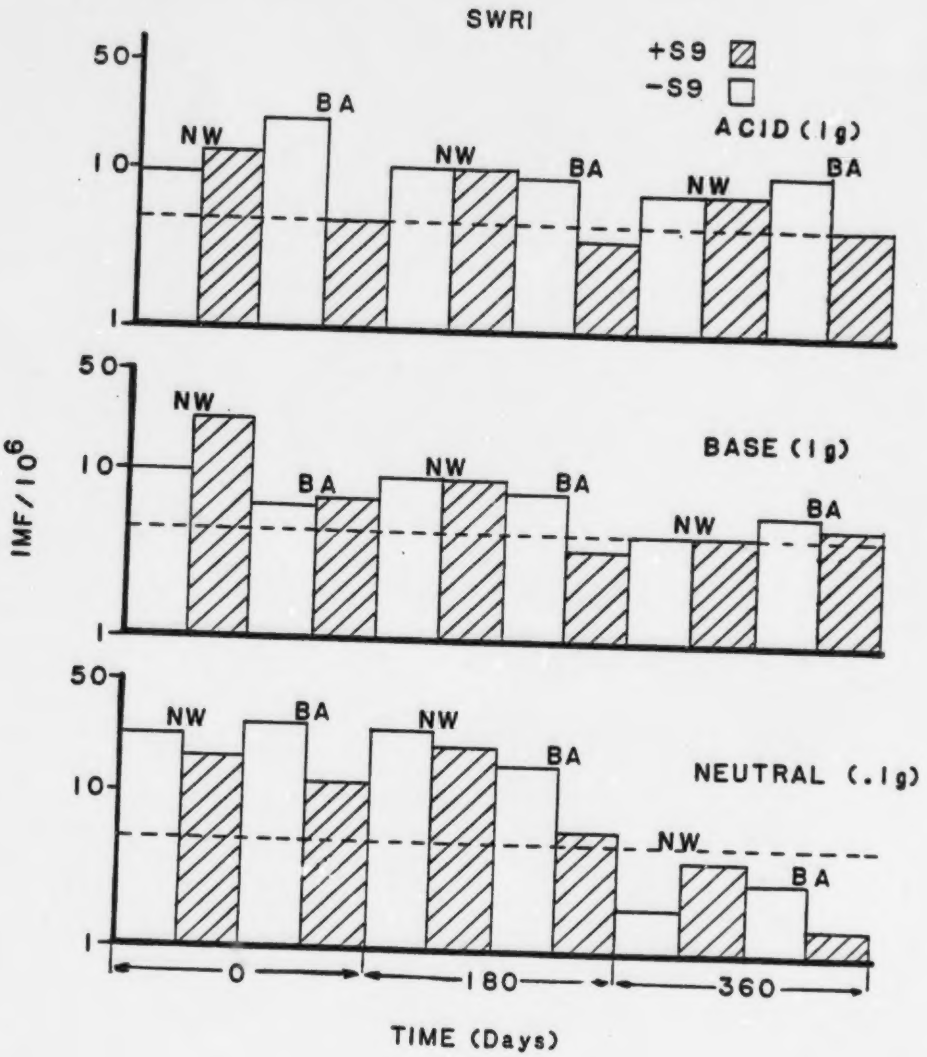

Figure 136. Total induced mutation frequency of equivalent volumes of SWRI amended Norwood (NW) and Bastrop (BA) soils as measured in A. nidulans methionine system with and without metabolic activation. Dashed line (---) is equal to total induced mutation frequency of $5.0/10^6$ survivors.

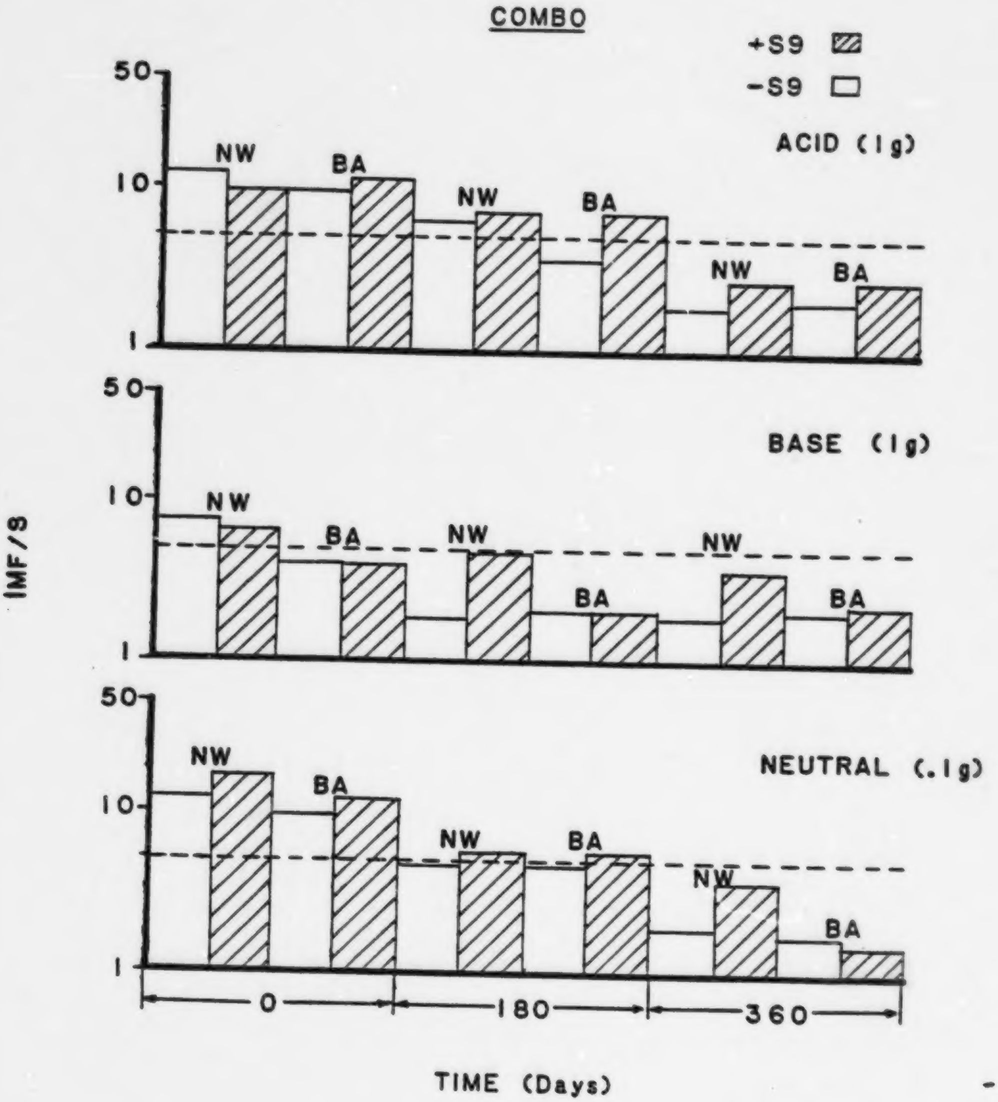

Figure 137. Total induced mutation frequency of equivalent volume of COMBO amended Norwood (NW) and Bastrop (BA) soils as measured in A. nidulans methionine system with and without metabolic activation. Dashed line (---) is equal to total induced mutation frequency of $5.0/10^6$ survivors.

fractions less or non-hazardous. However, the results also indicate that the neutral fraction, which represents greater than 90% of the total extractable hydrocarbons, was rendered non-hazardous by degradation in the soil.

The average rate of degradation of the COMBO waste in the Bastrop and Norwood soils was the maximum rate observed in the three wastes studied. This degradation rate was also reflected in the mutagenic potential of the soil extracts as measured in the biological systems. The response induced by the acid, base, and neutral fractions extracted from the COMBO waste amended Norwood soil collected on day 360 was less than a level at which the sample would be considered mutagenic in both the Salmonella and Aspergillus assays (Figures 134 and 137). Similarly, in the waste amended Bastrop soil the response induced by the fractions of the samples collected on day 360 was less than that induced by the samples collected on day 0. In addition, except for the acid fraction from the Bastrop soil in the Salmonella assay, the mutagenic potential of the three waste fractions from day 360 were less than the significant level in both bioassays. Thus, if the response in the eukaryotic assay is assumed to provide a more accurate prediction of the mutagenic response in a mammalian system, all three fractions of the COMBO waste were rendered non-hazardous by land treatment.

The major factors influencing the mutagenic potential of the hydrocarbons residual at a land treatment facility include the number of different compounds present, the concentrations of those compounds, and the toxic effects and interactions of those compounds. Biodegradation will influence the mutagenic potential as it reduces the concentration of certain compounds and alters the reactivity of others. Oxidation or substitution at specific sites have been shown to increase the reactivity of PAH (LaVoie et al., 1981; Hubermann et al., 1978; Ho et al., 1981). The rate of biodegradation may increase as soil bacteria adjust to the types of hydrocarbons in the soil. Poglazova et al. (1967), Shabad et al. (1971) and Khesina et al. (1969) observed that the degradation of PAH by soil microorganisms was significantly increased in a soil previously exposed to these compounds. It is also anticipated that bacteria capable of degrading the more complex PAH will occur in nature, since compounds such as napthalene, phenanthrene, fluoranthene, dibenzothiophene, carbazole, and dibenzofuran are components of coal (Sims and Overcash, 1983). Soil microorganisms possess a broad range of enzymatic capabilities and should be capable of degrading an assortment of hazardous compounds (Clark et al., 1979; Patil et al. 1970; Chacko et al., 1966; Bixby et al., 1971). The results of this research demonstrate that land treatment can reduce the hazardous characteristics of a waste by transformation or degradation. Thus, land treatment can render a waste less or non-hazardous if it can also be demonstrated that hazardous constituents are immobilized in the soil.

SECTION 10

SOIL MOBILITY AND DEGRADATION OF MUTAGENIC CONSTITUENTS FROM A WOOD-PRESERVING BOTTOM SEDIMENT

INTRODUCTION

The interaction of environmental parameters with soil and waste components will greatly influence the rate at which hazardous organic constituents are transformed, degraded, or immobilized at a land treatment facility. While the majority of hazardous organic constituents will apparently be retained and degraded in the surface layer of soil, there is concern about the environmental fate of those constituents which may be mobile in soil. Mobile compounds may be soluble in water and move with the wetting front or they may be hydrophobic and move ahead of the wetting front. In addition, the mobility of a compound may be modified by chemical and biological reactions occurring in the soil.

In order for land treatment to be an environmentally sound alternative for hazardous waste disposal, there is a need to develop procedures for sampling and techniques for monitoring mobile compounds in soil. Two procedures have been recommended for sampling mobile compounds at a hazardous waste land treatment facility (EPA, 1982). Soil core samples are intended to provide information on the movement of "slower moving" constituents, whereas soil-pore samples are intended to provide information on the movement of fast-moving hazardous constituents that may be missed by a soil-core sample. This research project was designed to provide information as to the utility of the two sampling procedures and to monitor the mobility of the components of a complex mixture in soil using both biological and chemical analysis.

MATERIALS AND METHODS

Soil

Undisturbed soil monoliths of the Norwood silt loam were used in this study. The properties of this soil are discussed in detail in Section 6, and the chemical and physical properties with depth are provided in Table 93. The soil monolith was obtained from a virgin area that had not received waste applications of any kind.

TABLE 93a. CHEMICAL PROPERTIES OF THE NORWOOD SOIL SERIES

Depth (cm)	pH	CEC* (meq/100 g)	Exchangeable Cations Ca^{++}	Mg^{++}	K^{+}
			------ meq/ 100 g --------		
0.0-15.2	7.69	19.6	20	4.0	0.8
15.2-30.5	7.73	21.1	20	4.0	0.8
30.5-61.0	7.74	22.9	20	4.0	0.8
61.0-91.5	7.86	17.8	20	4.0	0.8
91.5-119.0	7.75	16.2	20	4.0	0.8
119.0-122.0	7.75	30.0	20	4.0	0.8
122.0-152.0	7.95	13.2	20	4.0	0.8

*CEC = cation exchange capacity.

TABLE 93b. PHYSICAL APROPERTIES OF THE NORWOOD SOIL SERIES

Depth (cm)	Texture (USDA)	Particle Distribution Sand	Silt	Clay
		--------------- (%) -----------		
0.0-15.2	sc*	48.2	15.2	36.6
15.2-30.5	scl	49.6	15.1	35.3
30.5-61.0	c	36.0	18.4	45.6
61.0-91.5	cl	40.5	22.3	37.2
91.5-119.0	cl	42.0	25.0	33.0
119.0-122.0	c	23.1	20.5	55.6
122.0-152.0	scl	49.1	15.8	35.1

* sc = sandy clay, scl = sandy clay loam, c = clay, cl = clay loam

Waste

The waste used in this segment of the research was a wood-preserving bottom sediment. A detailed description of this waste is given in Section 5 and the characteristics summarized in Table 50.

Lysimeters

Six undisturbed soil monolith lysimeters were used in this portion of the study. A detailed description of the procedures for the collection and installation of lysimeters is given by Brown *et al.* (1974). Briefly, the technique is as follows: Casings (28 cm x 90 cm) were obtained from the Sharney Container Corp. (Houston, Texas). These 55 gal straight-walled barrels were cleaned and coated with an epoxy based heavy metal free paint (Shertar, Sherwin-Williams Co). Using an iron frame for support, the casings were forced into the ground with pressure applied by a backhoe. As the casing was pushed into the ground, the soil was removed around the outside to relieve the pressure. The backhoe was used to lift the soil monolith and to roll it upside down. Sufficient soil was removed from the bottom of the profile to install three porous ceramic suction cups at the bottom of the profile. The nylon tubes that were used to conduct the leachate to the surface were threaded through a 1.27 cm diameter PVC tube installed along the inside of one wall. A barrel gasket was sealed in place, and the bottom was clamped on the barrel. The lysimeters were then turned upright and installed in a location to facilitate leachate collection.

Leachate Sample Collection

A schematic diagram of the leachate collection system is shown in Figure 138. Leachate from the bottom of each lysimeter was collected through 3 porous cups (Coors Type #70001-P-6-C). Each had a bubbling pressure of 0.5 bars, a conductivity of 1.2 ml cm^{-2} min^{-1} bar^{-1}, and a surface area of 38.8 cm^{2}.

Plexiglass caps were sealed to the open end of the cups with a water proof two part epoxy (Armstrong #34). Nylon tubes .159 cm in diameter were cemented into the plexiglass caps with another two part epoxy (Armstrong #6) that adhered well to nylon. The three tubes passed through the 1.27 cm PVC pipe to a plexiglass collection manifold approximately 7.5 cm above the soil surface. Nylon tubes of 0.32 cm diameter were used to convey the leachate to amber glass bottles in a refrigerator at 4°C. A continuous vacuum was maintained on all six bottles. Each bottle was equipped with an outflow tube connected to a trap bottle. A glass tube 0.64 cm in diameter was placed in the center of a rubber stopper in the trap bottle and connected to a vacuum pump by rubber tubing. Leachate samples were collected as necessary by replacing the amber glass bottles. Leachate samples from each lysimeter were composited for the thirty days prior to waste application, day 0 to 30 after application, and day 30 to 90 after application. All containers

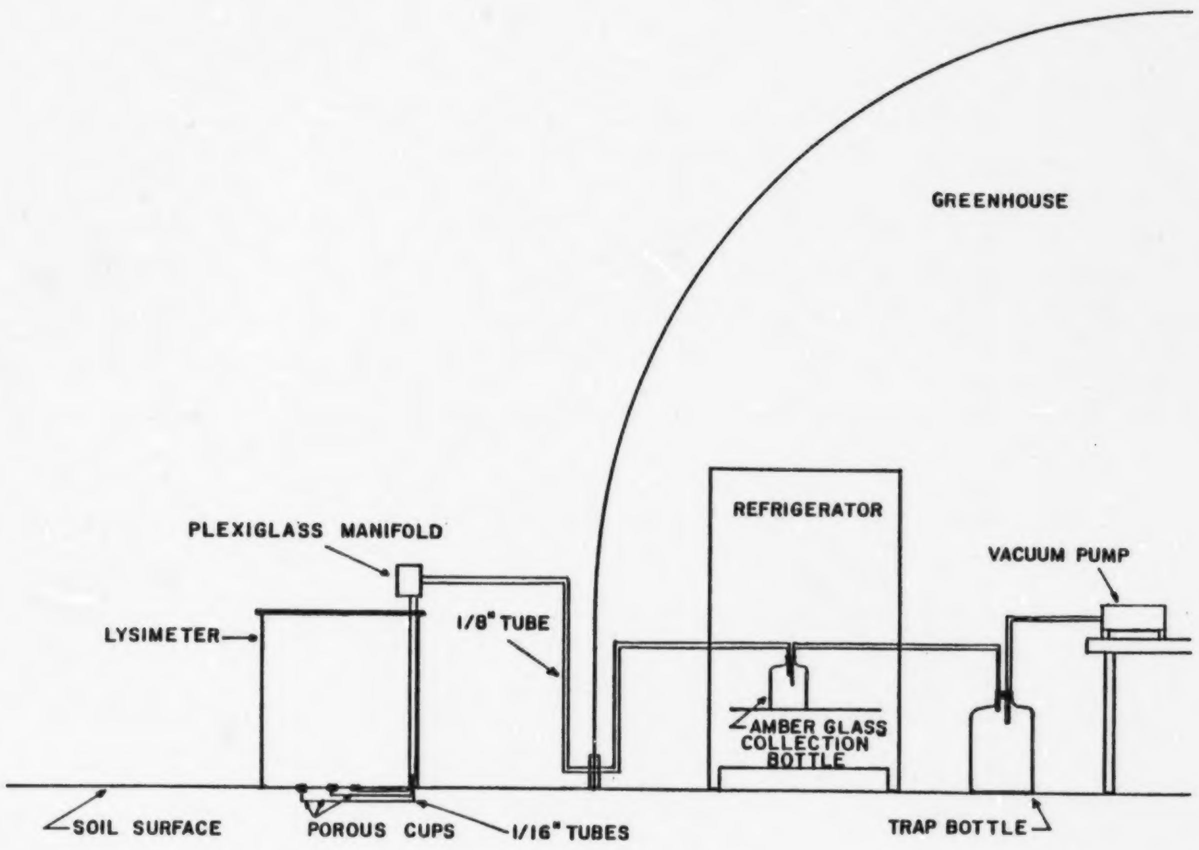
GREENHOUSE
PLEXIGLASS MANIFOLD
LYSIMETER
1/8" TUBE
REFRIGERATOR
VACUUM PUMP
AMBER GLASS COLLECTION BOTTLE
SOIL SURFACE
POROUS CUPS
1/16" TUBES
TRAP BOTTLE

Figure 138. Schematic diagram of leachate collected system used in the lysimeter study.

were cleaned with soap and water and sequentially rinsed with tap water, acetone, and distilled water to remove all organics prior to sampling. All samples were stored at 4°C after arrival at the laboratory.

Soil Sample Collection

Surface soil samples, 0 to 15 cm, were collected from each lysimeter prior to and immediately after waste application. Ninety days following waste application, the lysimeters were dissected using a circular saw equipped with an abrasive blade. Four of the six lysimeters were sacrificed in order to collect the amount of sample needed. Sufficient soil was collected to conduct a biological and chemical analysis on the soil extract, as well as to provide a reserve sample for future use. Soil samples were collected from the center of each lysimeter at depths of 0 to 15 cm, 15 to 45 cm, and 45 to 90 cm. Precautions were taken to prevent cross-contamination of cores. These included collecting the soil-cores from the center of the lysimeter to avoid any influence due to side-channel flow, cleaning the sampling device between corings, collecting samples from control before waste-amended lysimeters, and collecting multiple composited samples from each lysimeter at each depth.

Biological Analysis

The ability of the organic extract of soil and water samples to induce genetic damage was measured in a prokaryotic system capable of detecting compounds which induce point mutations. The *Salmonella*/microsome assay of Ames *et al*. (1975) utilizes a prokaryotic organism to evaluate the capacity of a sample to induce reverse mutations to histidine prototrophy. *Salmonella* strain TA98 (a frameshift mutant) was supplied by Dr. B. N. Ames (University of California, Berkeley, CA). The procedural methods were the same as Ames *et al*. (1975), except that overnight cultures were prepared by inoculation into 10 ml of Nutrient Broth #2 (KC Biological, Inc., Lenexa, KS). Soil extracts were tested on duplicate plates in two independent experiments in the standard plate incorporation assay at a minimum of 4 dose levels of the sample with and without enzyme activation (0.3 ml rat liver/ml S-9 mix). Aroclor 1254 induced rat liver was obtained from Litton Bionetics (Charleston, SC). Positive controls included 25 µg/plate 2-nitrofluorene (Aldrich Chemical Co., Milwaukee, WI), and 10 µg/plate 2-aminoanthracene (Sigma) which was used to verify the functioning of the metabolic activation system. All reagents and extracts were tested for sterility; DMSO was used as a negative control.

RESULTS AND DISCUSSION

The chemical and biological analysis of soil and leachate water from control and waste amended lysimeters indicate that certain constituents of the wood-preserving bottom sediment are capable of migrating through the soil. The analysis of soil-core samples from control and waste-amended lysimeters over various depths indicated that greater quantities of residual hydrocarbons and mutagenic activity were present in waste amended samples up to a depth of 45 cm (Figure 139). There was no appreciable difference in the mutagenic potential of the soil from control and waste amended lysimeters at the 45 to 90 cm depth, although greater quantities of residual hydrocarbons were recovered from the waste amended soil at this depth. The mutagenic potential of the soil-core sample from a depth of 0 to 15 cm was greatest in the presence of metabolic activation; whereas, the soil at 15 to 45 cm gave approximately the same response with or without metabolic activation (Figure 139).

The results from the chemical analysis of soil-core samples from control and waste amended lysimeters are provided in Tables 94 and 95 and Figures 140, 141, 142, and 143. The compounds identified in the soil-core sample from the unamended lysimeter (Table 94 and Figure 140) were predominantly the same as those identified in the Norwood soil (Table 45 and Figure 52). Two compounds of an unknown origin were identified including 1,2-dichlorobenzene and trimethyl-hexadione. A larger number of compounds were identified in the waste amended soil at all depths than were identified in the unamended soil (Table 95 and Figures 141, 142, and 143). These included several high molecular weight polycyclic aromatic hydrocarbons. Mutagenic compounds identified in the surface soil sample (Figures 135, 136, and 137) included methylnapthalene, dihydro acenapthylene, fluoranthene, pyrene, and benzanthracene (Kaden _et al_., 1979; McCann _et al_., 1975). Other compounds with a potential influence on the genetic activity of the soil extract included pentachlorophenol, several alkane promotors and cocarcinogens, and hexadecane which is an inhibitor. Only one compound, dihydro acenapthylene, was consistently identified in the waste and soil core samples at all depths (Figure 94).

When compared to the soil-core samples, the analysis of soil-pore samples provides a slightly different perspective on the capacity of PENT S waste samples to migrate through the soil. Biological analysis indicated that there was no mutagenic activity, as measured with _S. typhimurium_ strain TA98, detected in the leachate water collected from control and waste amended lysimeters prior to waste application. The mutagenic potential of the soil-pore sample collected on day 30 was approximately seven times greater than the control, whereas the sample collected on day 90 had a mutagenic potential approximately ten times the control (Figure 144). Thus, the bioassays detected significantly greater quantities of mutagenic activities in the soil-pore samples from the PENT S lysimeters both on day 30 and on day 90.

Nine compounds were detected in the soil-pore liquid sample from the unamended lysimeters (Table 96 and Figure 145) including benzene-dicarboxylic acid and chloro-octane. Neither of these compounds is known to have any genotoxic activity. There were ten compounds

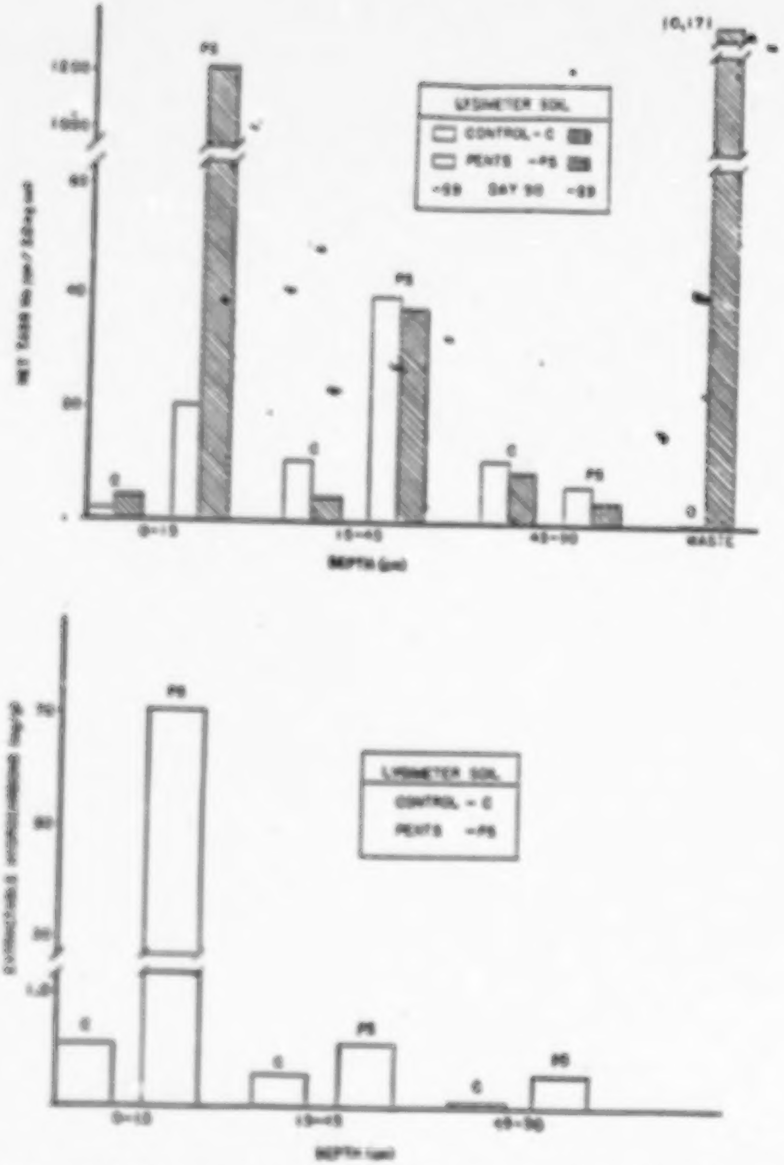

Figure 139. Extractable hydrocarbons and mutagenic activity from soil core samples collected at various depths on day 90.

TABLE 94. LIST OF COMPOUNDS DETECTED IN SOIL CORE SAMPLE COLLECTED ON DAY 90 FROM CONTROL NORWOOD SOIL AT 0-15 CM DEPTH

Compound (Peak Number)*	Genetic Toxicity[1]
Pentadecane (324)	X
Unknown (351)	X
Hexadecane (367)	A
Unknown (388)	X
Heptadecane (411)	X
Octadecane (443)	P; CC
Unknown (463)	X
Nonadecane (475)	X
Unknown (496)	X
Eicosane (507)	CC
Heneicosane (538)	X
Unknown (550)	X
1,2-dichlorobenzene (564)	M1
Trimethyl-hexanedione (599)	X
Unknown (614)	X
Unknown (663)	X
Unknown (684)	X

* Potential genetic toxicity: I = initiator; P = promotor; A = antagonist; CC = cocarcinogen (synergist); MO = nonmutagenic; M1 = mutagenic; CO = non-carcinogen; C1 = carcinogen; all others = no information. The references used in determining these factors are listed in the text.

TABLE 95. LIST OF COMPOUNDS DETECTED IN SOIL CORE SAMPLES COLLECTED ON DAY 90 FROM PENT S WASTE AMENDED NORWOOD SOIL AT VARIOUS DEPTHS

Sample	Fraction		
	Acid Compound (Peak Number)*	Base Compound (Peak Number)*	Neutral Compound (Peak Number)*
0-15 cm	Unknown (222)	Tridecane (249)	Unknown (242)
	Tridecane (238)	Dimethyl napthalene (296)*MO	Unknown (304)
	Methyl napthalene (243)*Ml; CO	Tetradecane (311)*P	Pentadecane (338)
	Unknown (262)	Dihydro acenaphthalene (323)*Ml	Dimethyl napthalene (351)*MO
	Tetradecane (283)*P	Unknown (337)	Dihydro acenaphthylene (366)*Ml
	Dimethyl napthalene (290)*MO	Pentadecane (353)	Dibenzofuran (375)
	Dihydro acenaphthalene (317)*Ml	Phenalene (366)*Ml	Trimethyl naphthalene (385)*MO
	Pentadecane (330)	Hexadecane (384)*A	Heptadecane (401)
	Phenalene (369)*Ml	Carbazole (402)*MO; CO	Methyl dibenzofuran (416)
	Hexadecane (379)*A	Heptadecane (414)	Octadecane (437)*P; CC
	Unknown (396)	Octadecane (445)*P; CC	Dibenzothiophene (455)*MO
	Heptadecane (409)	Unknown (457)	Nonadecane (472)
	Pentachlorophenol (437)*MO; Cl	Nonadecane (477)	Eicosane (506)*CC
	Methyl dibenzothiophene (461)*MO	Cyclopentaphenanthrene (485)*MO	Ethyl undecane (520)
	Nonadecane (472)	Eicosane (509)*CC	Methyl hexadecane (540)
	Eicosane (503)*CC	Fluoranthene (530)*Ml; CO; CC	Docosane (569)
	Fluoranthene (525)*Ml; CO; CC	Heneicosane (540)	Branched alkane (595)
	Pyrene (540)*Ml; CO; CC	Pyrene (545)*Ml; CO; CC	Tricosane (620)
	Docosane (563)	Docosane (569)	Tetracosane (639)
	Unknown (592)	Unknown (599)	Benzophenanthrene (659)
	Unknown (620)	Unknown (628)	Benzanthracene (680)*Ml; Cl
	Unknown (642)		
	Unknown (671)		

TABLE 95 CONTINUED

15-45 cm (crude) Compound (Peak Number)*	45-90 cm (crude) Compound (Peak Number)*
Unknown (213)	Unknown (230)
Tridecane (234)	Unknown (257)
Unknown (267)	Dihydro acenaphthylene (307)*Ml
Tetradecane (277)*P	Dimethyl nonane (314)
Unknown (303)	Unknown (339)
Dihydro acenaphthylene (311)*Ml	Trimethyl octane (354)
Pentadecane (319)	Unknown (373)
Unknown (338)	Tetrazolamine (393)
Fluorene (351)*MO; CO	Unknown (475)
Hexadecane (358)*A	Benzenedicarboxylic acid (487)*MO
Trimethyl octane (378)	Methyl hexene (521)
Heptadecane (398)	Unknown (563) (589)
Octadecane (435)*CC	Ethyl methyl pentanol (604)
Unknown (450)	Ethyl methyl heptane (610)
Nonadecane (470)	Unknown (636)
Benzene dicarboxylic acid (492)*MO	Methyl heptanol (651)
Eicosane (502)*CC	Unknown (685) (716)
Unknown (514)(523)(554)(592)	Methyl propyl pentanol (738)
	Unknown (755) (780)

* Potential genetic toxicity: I = initiator; P = promotor; A = antagonist; CC = cocarcinogen(synergist); MO = nonmutagenic; Ml = mutagenic; CO = non-carcinogen; Cl = carcinogen; all others = no information. The references used in determining these factors are listed in the text.

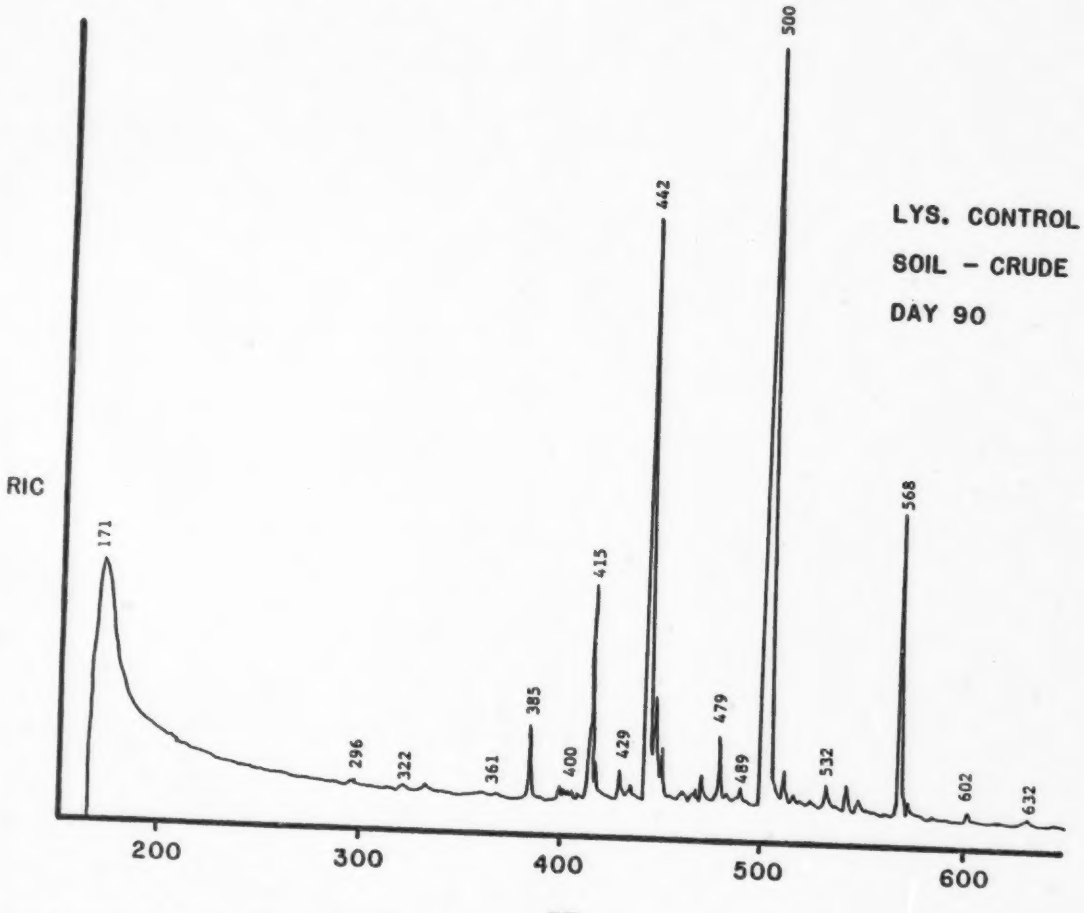

Figure 140. GC/MS chromatograph of crude extract of soil core sample collected on day 90 at a depth of 0 to 15 cm from unamended Norwood lysimeter.

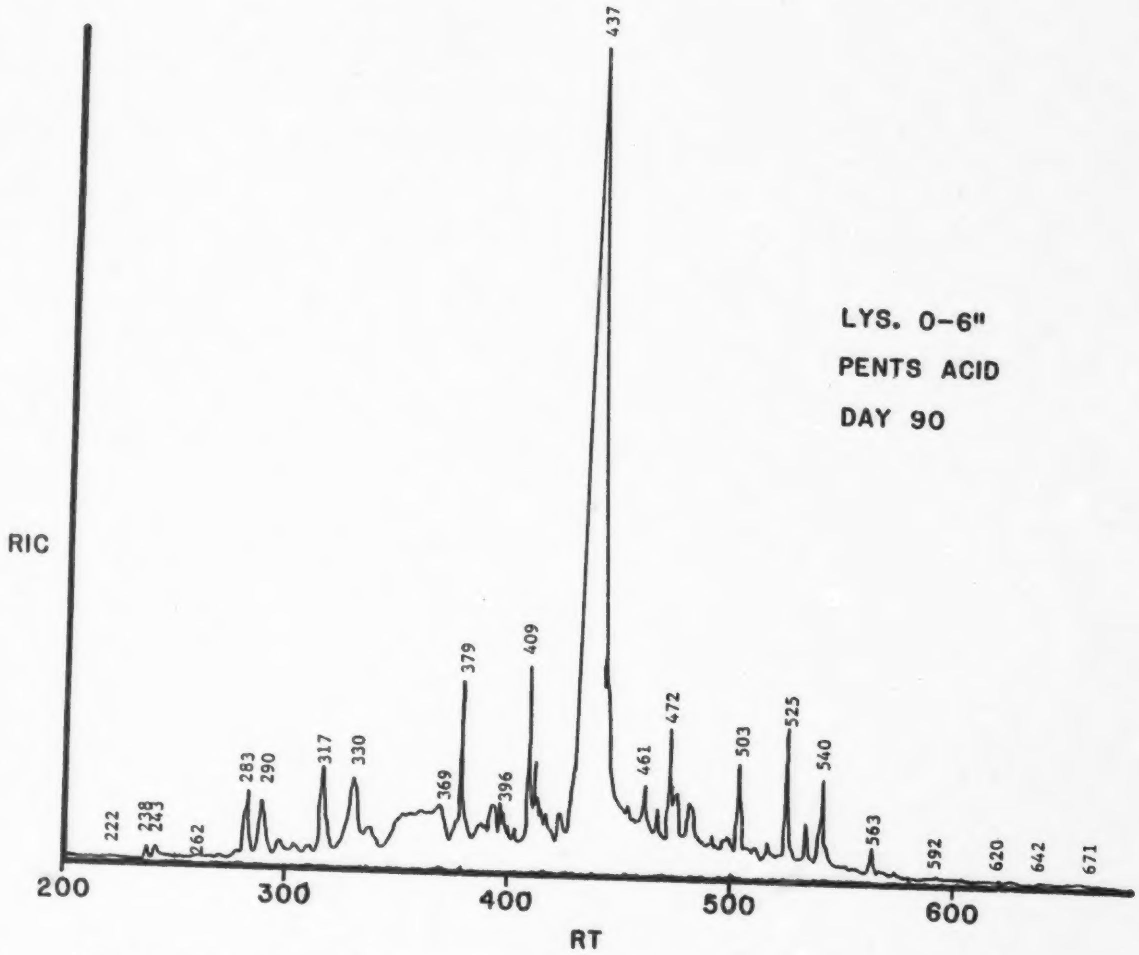

Figure 141. GC/MS chromatograph of acid fraction of soil core sample collected on day 90 at a depth of 0 to 15 cm from PENT S amended Norwood lysimeter.

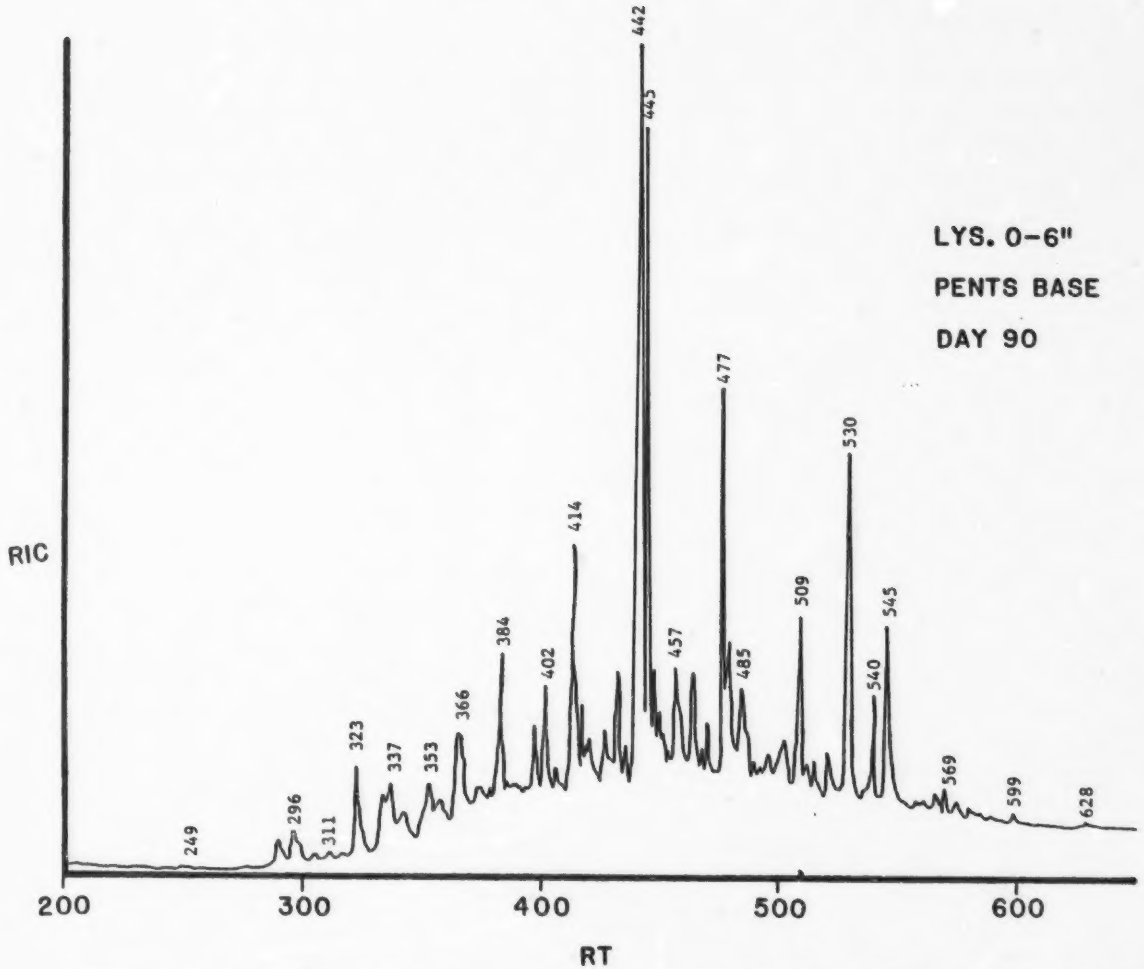

Figure 142. GC/MS chromatograph of base fraction of soil core sample collected on day 90 at a depth of 0 to 15 cm from PENT 3 amended Norwood lysimeter.

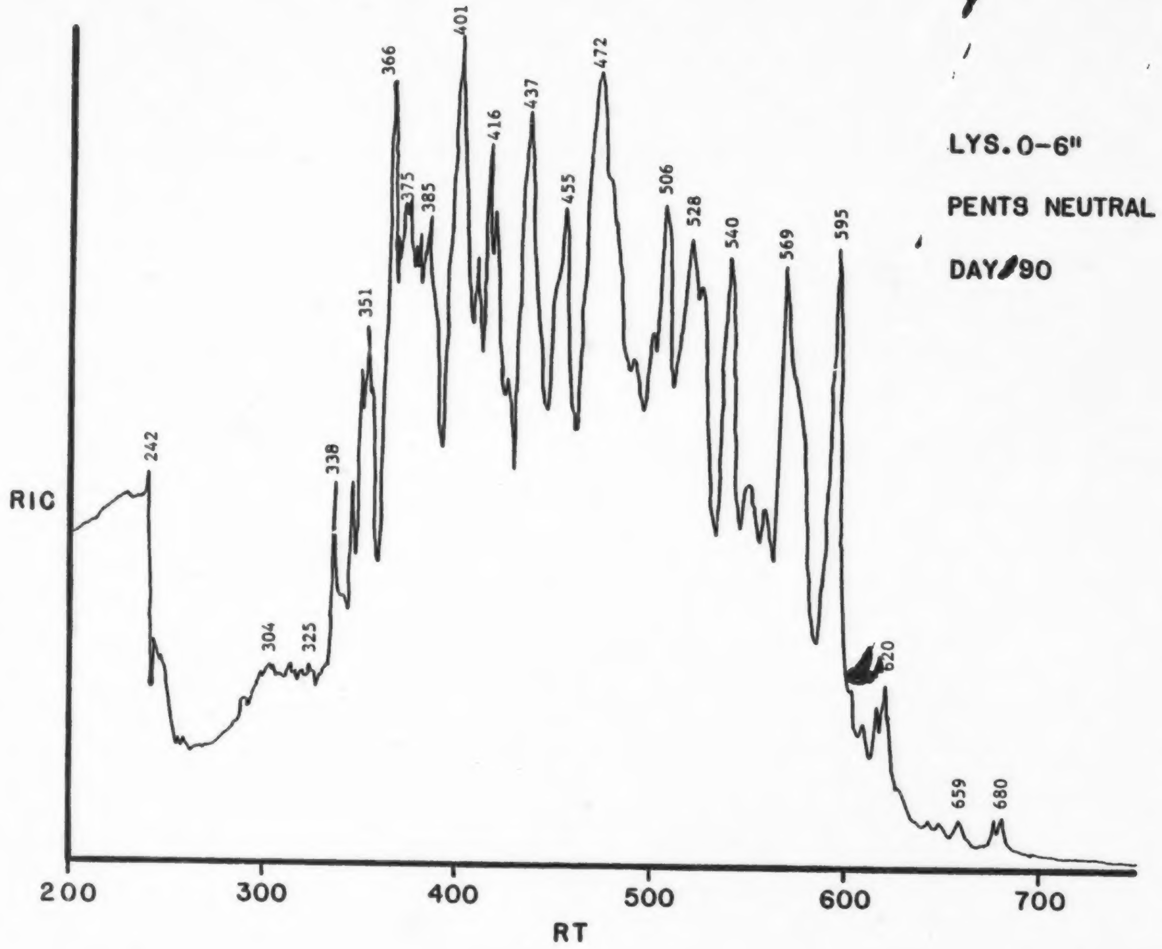

Figure 143. GC/MS chromatograph of neutral fraction of soil core sample collected on day 90 at a depth of 0 to 15cm from PENT S amended Norwood lysimeter.

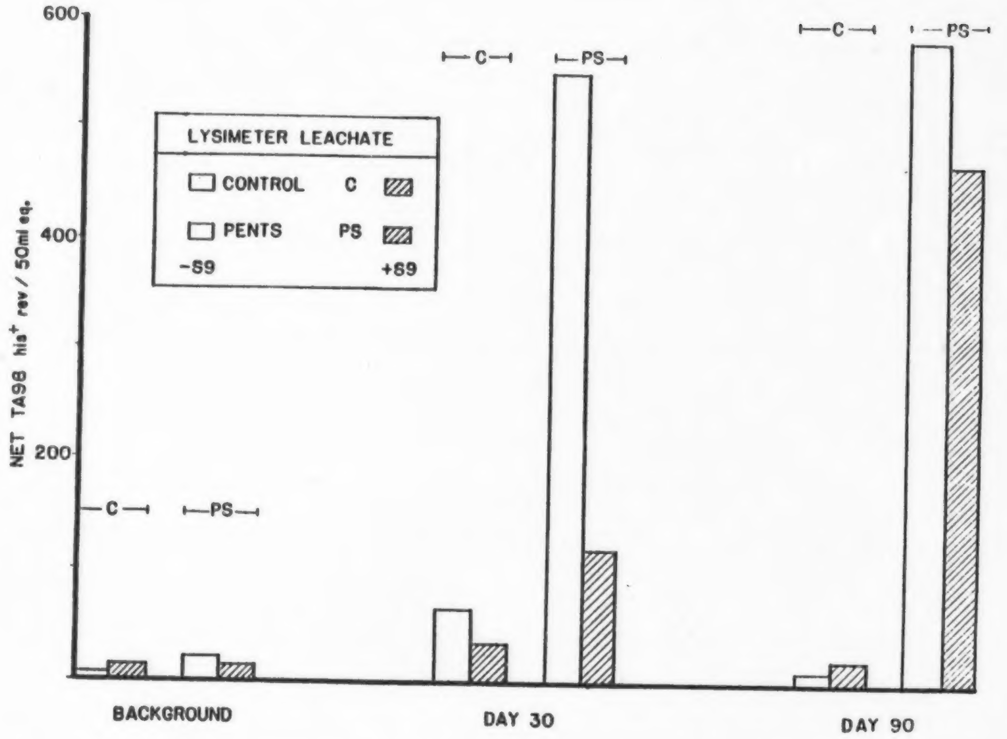

Figure 144. Mutagenic activity of leachate water from control and PENT S waste amended lysimeters.

TABLE 96. LIST OF COMPOUNDS DETECTED IN SOIL PORE SAMPLES COLLECTED ON DAY 90 FROM PENT S WASTE AMENDED NORWOOD SOIL

	Compound (Peak Number)*		Compound (Peak Number)*
PENT S	Unknown (164)		
	Hydroxyl methyl pentanone (185)	CONTROL:	Unknown (298)
	Unknown (212)		Unknown (391)
	Unknown (226)		Unknown (417)
	Unknown (251)		Benzene dicarboxylic acid(491)*MO
	Unknown (278)		Unknown (542)
	Octanedione (287)		Unknown (572)
	Unknown (308)		Unknown (602)
	Unknown (325)		Chloro-octane (632)
	Tetrachlorophenol (338)#MO		Unknown (661)
	Phenyl butanone (362)		
	Phenalene (376)*M1		
	Anthracene (402)*MO		
	Pentachlorophenol (415) (429)*MO; C1		
	Benzene dicarboxylic acid (491)*MO		

* Potential genetic toxicity: I = initiator; P = promotor; A = antagonist; CC = cocarcinogen(synergist); MO = nonmutagenic; M1 = mutagenic; CO = non-carcinogen; C1 = carcinogen; all others = no information. The references used in determining these factors are listed in the text.

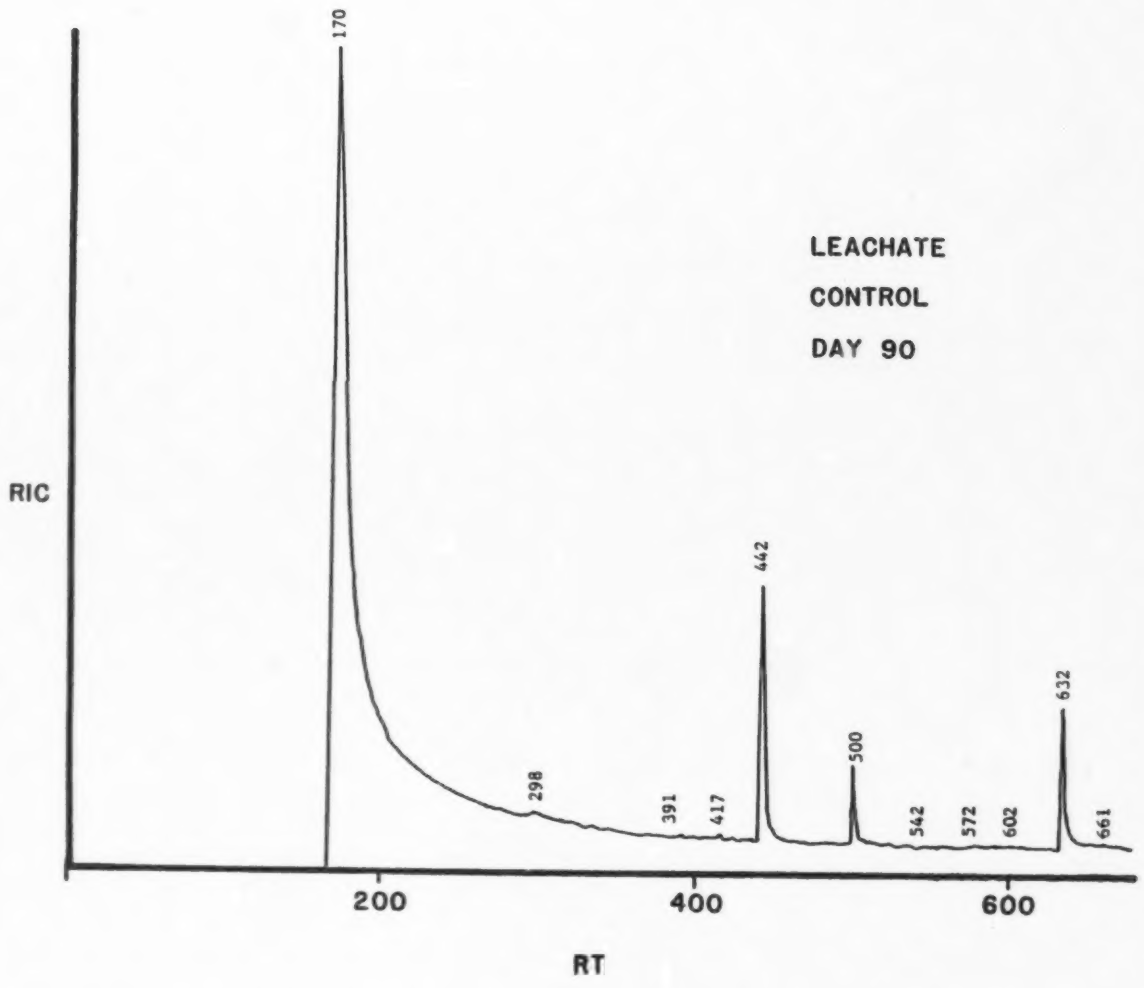

Figure 145. GC/MS chromatograph of soil pore liquid sample collected from unamended Norwood lysimeter on day 90.

identified in the soil-pore samples from the PENT S lysimeters, including anthracene and pentachloraphenol which were also detected in the 0 to 15 cm soil-core sample from day 90 (Table 96 and Figure 146). Anthracene, a non-mutagenic hydrocarbon, has been found to produce significant enhancement when in mixture with other polycyclic aromatic hydrocarbons (Hermann, 1981); at high concentrations, anthracene caused significant enhancement of 2-aminoanthracene (Kawalek and Andrews, 1981). Phenalene, another compound identified in a leachate sample, was also present in the waste but was not detected in any of the soil-core samples. Tetrachlorophenol, a degradation product of pentachlorophenol (Fishbein, 1977), was also detected in the 90 day leachate sample. This compound was not present in waste or soil-pore samples. As a result, the tetrachlorophenol may have been transformed from pentachlorophenol at the soil water interface and was subsequently leached into the soil-pore water 90 cm below the soil surface. The absence of tetrachlorophenol in the 0 to 15 cm soil sample makes it unlikely that the compound reached the soil-pore sample as a result of side channel flow.

This analytical protocol, using both biological and chemical analysis, has demonstrated that soil-core and soil-pore liquid monitoring can be used to detect different types of compounds. Mutagenic activity was detected in both soil-core and soil-pore samples, although no activity was detected in the 45 to 90 cm soil-core sample. Saturated alkanes, branched alkanes, and alkanols were identified in soil-core samples, while the more complex compounds such as tetra- and pentachlorophenol, phenalene, and anthracene were identified in the soil-pore samples. In addition, the results from utilizing bioassay directed chemical analysis indicated that compound(s) such as anthracene may have served to enhance the activity of mutagenic compounds present in the leachate in trace quantities. This combined testing protocol has demonstrated that both soil-core and soil-pore samples are necessary to provide an accurate evaluation of the potential for hazardous waste constituents to migrate below the zone of incorporation at a land treatment facility. The analysis of samples from lysimeters amended with a wood-preserving bottom sediment indicated that mutagens and potential carcinogens are capable of migrating to a depth of 75 cm below the zone of incorporation within 90 days following waste application. These results indicate that land treatment of a wood-preserving waste should proceed with caution, and that pretreatment options or in-plant process controls might be used to reduce the concentration of mobile constituents in the waste prior to land application.

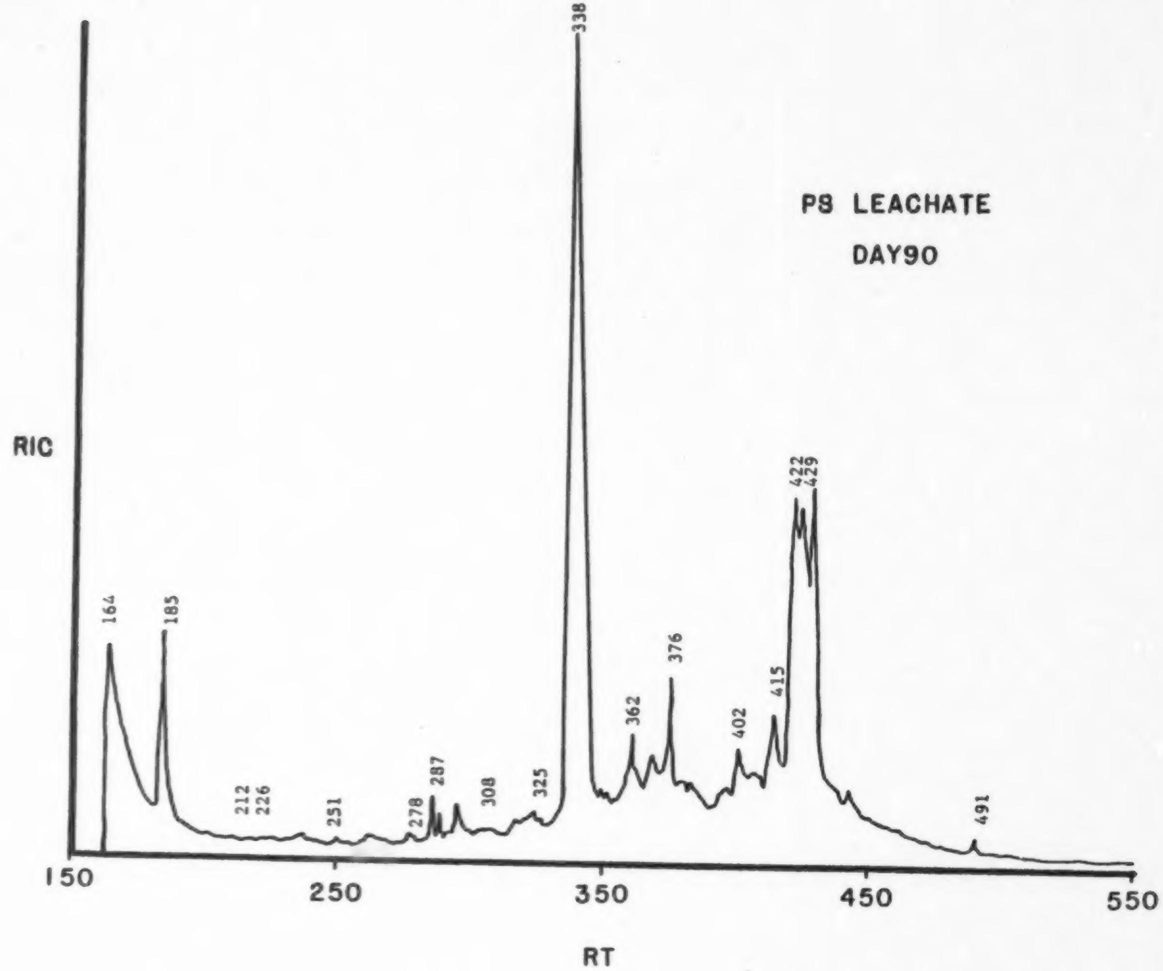

Figure 146. GC/MS chromatograph of soil pore liquid sample collected from PENT S amended lysimeter on day 90.

SECTION 11

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